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SYMPTOMATIC PARTIAL PARALYSIS IN CHICKS INFECTED WITH *PLASMODIUM JUXTANUCLEARE**

By

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INTRODUCTION

The involvement of the nervous system in human malaria (particularly *falciparum* malaria) gives rise to a well-known clinical entity. The symptoms which suggest such involvement however are not consistent and vary from case to case; cerebellar ataxia, hyperactive tendon reflexes, tremor, ataxia and dysmetria of the extremities, transitory paralysis and complete flaccid paralysis are among the symptoms which have been described as occurring in cerebral malaria (Rigdon, 1944; Rigdon and Fletcher, 1945; Turner, 1936; Wright, 1941; Masson, 1924; Anderson, 1927). Neurological manifestations have rarely been observed in animals experimentally infected with malaria. Simpson (1944) noticed partial paralysis and inability to raise the head or to stand in turkeys infected with *P. durae*. Huff (1957) states that turkeys infected with blood-induced *P. lophurae* or blood-induced *P. fallax* exhibit, prior to death, symptoms which suggest the involvement of the central nervous system, but he has not described them. Fletcher and Rigdon (1946) also describe the development of clinical manifestations, such as ataxia, inco-ordination and disturbance of posture in ducks infected with *P. lophurae*.

The present work describes nervous manifestations associated with *P. juxtanucleare* in chickens.

MATERIALS AND METHODS

The strain of *P. juxtanucleare* used was brought from Brazil by Professor P. C. C. Garnham in December, 1955 and was maintained in the Department of Parasitology, London School of Hygiene and Tropical Medicine by blood or spleen emulsion inoculation until received by the writer in February, 1957. The chicks used were obtained when one day old from a commercial firm. They were Rhode Island Red or Light Sussex. The chicks were infected, unless otherwise stated, when 1 to 7 days old. They were kept on a formulated diet, a baby chick mash with added vitamins. Green food was given once or twice a week, and small amounts of grit were supplied to birds once a week. Chicks were infected by the intraperitoneal injection of 0·5cc. of citrated blood taken from a bird, usually at the peak of parasitaemia, by puncturing the brachial vein or one of its branches. Organ suspensions were used after removing all traces of blood from the organs by a small piece of blotting paper, cutting into small pieces and triturating in a homogenizer (Griffith's tube) with 2 to 5cc. of physiological saline solution.

The birds were inoculated with 0·5cc. of the resultant suspension.

* This work forms part of a thesis approved for the Ph.D. Degree of the University of London.

RESULTS

Symptoms

Partial paralysis was observed in 10 out of 148 (6.7 per cent) chicks infected with *P. juxtanucleare* (Table 1). The symptoms exhibited by chicks showing this paralytic syndrome developed 5 to 12 weeks after the blood-induced *P. juxtanucleare* infection. All chicks showing the symptoms died in a state of severe exhaustion and emaciation within 3 to 10 days. The parasite count in the peripheral blood of these chicks was always high, since 10 to 15 per cent. of erythrocytes were infected. The sequence of events in this paralytic syndrome was as follows: The chicks at first show inco-ordination of movement and weakness of the legs. They may eventually become absolutely incapable of moving, and then usually crawl or move in a squatting position when disturbed. The paralysis does not extend to the neck or wings. The syndrome is usually associated with systemic disturbances, such as loss of weight and appetite, emaciation and pallor.

The pathological lesions observed in the organs of the paralysed chicks are more or less similar to those found in other chicks dying from chronic *P. juxtanucleare* infections. In general the lesions observed in the brain and spinal cord of paralysed chicks are more severe and pronounced than those noticed in other infected chicks. Diffuse gliosis and/or granulomata-like lesions are always present in the brains of chicks showing the paralytic syndrome. It should be mentioned in this connection that several chicks which died from chronic *P. juxtanucleare* infections without showing signs of paralysis have shown diffuse gliosis and/or granulomata-like lesions in their brains comparable to those noticed in the paralysed chicks.

Exclusion of Other Possible Causes of Lesions

A brain emulsion of a chick showing typical symptoms and lesions was injected intraperitoneally into two mice, according to the method of Lainson (1955). Both these mice and subsequent subinoculated mice survived and none showed toxoplasmosis.

Another experiment (Table 1) was designed to exclude concomitant viral or bacterial diseases, particularly viruses of avian encephalomyelitis and the avian leukosis complex. None of the chicks injected by the methods described showed the symptoms noticed in chicks infected with *P. juxtanucleare*. No malarial parasites were demonstrated in these experimental chicks throughout the 64 days of observations, when the chicks were killed, and no brain or heart lesions were observed in any of this group of chicks. The results of the experiments suggest that the paralytic syndrome is caused, most probably, by the effect of *P. juxtanucleare* itself on the infected chicks.

Seven out of the 21 chicks of this series died 1 to 19 days after the beginning of the experiment. At post mortem, none of them showed malarial parasites or lesions in the brain or heart, comparable to those seen in paralysed chicks. A similar high mortality rate was

TABLE 1

INOCULATION OF CHICKS

Sera and tissue emulsions (filtered and non-filtered) taken from chicks infected with *P. juxtanucleare* to exclude the presence of concomitant viral or bacterial infections

Group	*Number of chicks used*	*Age*	*Type of inoculum*	*Remarks*
I	3	2 days	Serum neither centrifuged nor diluted	One died after 6 days, one died after 19 days, the third killed after 64 days: none showed parasites, paralysis or brain lesions.
II	3	2 days	Serum diluted: 1 part serum in 10 parts broth saline-penicillin: no centrifugation	One died after 1 day, one died after 4 days, the third killed after 64 days: none showed parasites, paralysis or brain lesions.
III	3	2 days	Brain and spleen emulsions centrifuged for 10 minutes at speed of 4,000 r.p.m. not diluted.	One died after 14 days, the other two killed after 64 days: none showed parasites, paralysis or brain lesions.
IV	3	2 days	Serum centrifuged for 10 minutes first at 4,000 r.p.m. then at 25,000 r.p.m.; filtered through 1-1. 3μ filter.	None died, all killed after 64 days: none showed parasites, paralysis or brain lesions.
V	3	2 days	Brain and spleen emulsion treated as in group IV	One died after 6 days, one died after 14 days, the third killed after 64 days: none showed parasites, paralysis or brain lesions.
VI	6	2 days	Serum + brain and spleen emulsion treated as in group IV; then filtered through membrane filter 100mμ pore size.	None died, all killed after 64 days: none showed parasites, paralysis or brain lesions.

All chicks were given 0.5cc. of inoculum intraperitoneally, taken from a chick showing typical symptoms and lesions.

noticed in all experiments in which very young chicks, 1 or 2 day old, were used, most probably because such young chicks are extremely delicate and mere handling may lead to their death.

DISCUSSION

Paralytic symptoms may develop in chicks in a wide variety of diseases which include tuberculosis, staphylococcosis, fowl cholera, helminthiasis, coccidiosis, botulism, lead poisoning, avian toxoplasmosis and avian leukosis complex (Erichsen and Harboe, 1953a, 1953b; Harboe and Erichsen, 1954; Jungherr, 1952; Manwell, Coulston, Binckly and Jones, 1945).

The lesions observed in the brain and spinal cord of chicks infected with *P. juxtanucleare* have a close similarity to avian encephalomyelitis, avian toxoplasmosis, avian leukosis complex and perhaps other conditions (Jungherr, 1952; Pappenheimer, Dunn and Cone, 1929; Furth, 1931; Jones, 1934; Cole, 1938; Blakemore, 1939; Emmel, 1936; McGaughey and Downie, 1930; Becker, 1952; Patterson, 1936; Merchant and Packer, 1956; Lainson, 1955; Ratcliffe and Worth, 1951; Harboe and Erichsen, 1954; Erichsen and Harboe, 1953a, 1953b). The general clinical and pathological picture presented by the paralysed chicks infected with *P. juxtanucleare* is therefore not characteristic. Differential diagnosis between this syndrome in *P. juxtanucleare* infections and the other diseases mentioned does not seem to be possible without finding the parasites in the blood. The clinical symptoms observed in the paralysed chicks can easily be explained on the basis of the pathological lesions found in the brain and spinal cord of the affected birds. Death in these cases is due, most probably, to paralysis of the "vagus centre" in the medulla.

CONCLUSIONS

Partial symptomatic paralysis observed in 10 out of 148 chicks infected with *P. juxtanucleare* may be due to the severe necrotic and inflammatory lesions observed in the brains and spinal cords of chicks showing such symptoms. The lesions observed in these birds are caused, most probably, by the effects of the malaria parasite and/or its by-products, on the central nervous system.

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RINDERPEST INTERFERENCE WITH CAPRINIZED RINDERPEST VIRUS

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INTRODUCTION

The so-called interference phenomenon has been well documented in recent years but in the late thirties interference between animal viruses was still a novel concept. In 1938 Pfaff (1940) vaccinated cattle with caprinized rinderpest virus. Two animals were inoculated with virulent rinderpest virus 24 hours later and although both developed rinderpest they recovered. Two other cattle resisted the effects of virulent virus given 48 hours after vaccination. Pfaff attributed the protection to the rapid development of immunity which he considered was serviceable at 24 hours and absolute in 48 hours. In the light of present knowledge a sounder hypothesis attributes the protection to interference of the virulent virus by the attenuated caprinized virus.

Many field veterinarians in Africa hold the entrenched belief that caprinized rinderpest virus has a therapeutic action in animals incubating or suffering from rinderpest. Edwards (1950) concurred and attributed the action to interference, but he failed to support his opinions experimentally.

The confirmation that caprinized rinderpest virus interferes with virulent rinderpest virus and that it lacks therapeutic action are herein reported.

MATERIALS AND METHODS

Caprinized rinderpest virus. The Kabete attenuated (KAG) strain of caprinized rinderpest virus was used. It has been employed widely and successfully in East Africa as a live virus vaccine. The virus was packed in vacuum-sealed ampoules in the form of powdered freeze-dried infected goat spleens. The ampoules were kept at refrigerator temperatures until required and the contents were reconstituted with saline just prior to inoculation.

Virulent rinderpest virus. The Mpwapwa (M) strain of virulent rinderpest virus in the form of suspensions of infected bovine spleen was used. Unexpectedly the strain proved to be relatively avirulent and the case mortality rate was only 10 per cent.

Cattle. Tanganyika Shorthorn Zebu steers were used. They had never been in contact with rinderpest and had never been vaccinated.

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Experimental design. The cattle were divided into eleven groups of four animals. Group A were given M virus alone, Groups B, C and D were given M virus followed by KAG 48, 24 and 13 hours later respectively. Group E were given M and KAG simultaneously. Groups F, G, H, I and J were given KAG followed by M virus 11, 24, 33, 48 and 57 hours later respectively and group K were given KAG alone. The dose of KAG was the routine field dose. The dose of M virus was equivalent to one gramme of wet spleen. Aliquots of the same bovine spleen were used throughout. At the end of the experiment the remaining spleen was titrated in cattle and proved to have a titre in excess of 10^{-6} ID_{50} per gramme.

The cattle were kept under conditions as nearly identical as possible. Rectal temperatures were taken every morning and the cattle were carefully examined at short intervals. Particular attention was paid to clinical signs which were assessed for severity by a point system (Table 1). The system was quite arbitrary but was based on the experiences of the authors. All surviving cattle were challenged three weeks later with virulent rinderpest virus.

Statistical analysis. The data were subjected to the Analysis of Variance and significant differences among groups were determined by the Multiple Range Test (Duncan, 1955).

RESULTS

Case Mortality Rate

Only four (9·1 per cent) cattle died. Two deaths occurred in cattle given M virus before KAG and two deaths occurred in cattle given KAG before M virus.

TABLE 1
ASSESSMENT SYSTEM

System	*Signs*	*Points*	*Maximum points*
Ocular			4
	Injected conjunctivae	2	
	Slight injection	1	
	Conjunctival catarrh	2	
	Ocular discharge	2	
	Slight ocular discharge	1	
Nasal			2
	Nasal discharge	2	
	Slight discharge	1	
Oral			5
	Erosions	3	
	Erythema of mucous membrane	1	
	Slight erythema of mucous membrane	0·5	
	Inflammation of gums	2	
	Erythema of gums	1	
	"Rinderpest smell"	2	
	Slight smell	1	
Intestinal			3
	Dysentery	3	
	Diarrhoea	2	
	Slight diarrhoea	1	
Death			14

TABLE 2

ASSESSMENT OF CLINICAL SIGNS

Group	*Treatment*	*Cattle*	*Clinical assessment*
A	M alone	410	8·0
		411	11·0
		412	8·0
		413	8·5
B	M 48 hours before KAG	414	11·0
		415	10·0
		416	14·0*
		417	5·0
C	M 24 hours before KAG	418	11·0
		419	8·0
		420	13·0
		421	12·0
D	M 13 hours before KAG	422	10·0
		423	8·0
		424	7·5
		425	14·0*
E	M and KAG simultaneously	426	5·0
		427	11·0
		428	8·5
		429	12·0
F	KAG 11 hours before M	430	11·0
		431	11·0
		432	14·0*
		433	11·0
G	KAG 24 hours before M	434	8·0
		435	7·0
		436	6·5
		437	8·0
H	KAG 33 hours before M	438	9·0
		439	5·5
		440	4·0
		441	14·0*
I	KAG 48 hours before M	442	7·0
		443	4·5
		444	1·5
		445	4·5
J	KAG 57 hours before M	446	7·0
		447	3·0
		448	3·0
		449	2·0
K	KAG alone	450	2·5
		451	4·5
		452	2·5
		453	1·0

* Died.

Reaction Assessments

The cattle given M virus alone had a point average of 8·875 with a standard deviation of 1·44 whereas the group given KAG alone had a point average of 2·625 with a standard deviation of 1·44. The difference was highly significant ($t = 6·158$, $P < 0·001$).

Dual Infections

Data are recorded in Table 2 and the Analysis of Variance is shown in Table 3. The Variance Ratio between treatments was 5·18 (d.f. 10, 30) which was highly significant ($P < 0·01$). The Variance Ratio between replicates was not significant.

TABLE 3

ANALYSIS OF VARIANCE OF THE CLINICAL ASSESSMENTS

Source	*Degrees of freedom*	*Sum of squares*	*Mean square*
Between treatments	10	371·98	37·198**
Between replicates	3	5·79	1·930
Error	30	215·52	7·184
Total	43	593·29	

The Multiple Range Test revealed that the group means fell into four series which differed significantly from each other (Table 4). The clinical assessments of the groups in the first series were indistin-

TABLE 4

GROUPS WITH SIGNIFICANTLY DIFFERENT CLINICAL ASSESSMENT MEANS

Series 1	*Series 2*	*Series 3*	*Series 4*
A	G	G	I
B	H	I	J
C	I	J	K
D			
E			
F			
G			
H			
9·516	6·625	5·167	3·583

guishable from the assessment made when M virus alone was inoculated. The groups included all those in which M virus was given before KAG, the group given M and KAG simultaneously and the groups in which KAG was given up to 33 hours before M virus.

The clinical assessments of the groups in the fourth series were indistinguishable from the assessment made when KAG alone was inoculated. The groups were those in which KAG was given 48 and 57 hours before M virus. The second and third series had assessments which fell between that of M virus alone and KAG alone. The groups were all those given KAG virus 24 to 57 hours before M virus. In other words, the second series overlapped the first and the third series overlapped the fourth.

Immunity

The surviving cattle proved to be immune when challenged three weeks later.

DISCUSSION

Despite the weakness occasioned by the virulent virus proving to be relatively mild which forced us to use an arbitrary assessment system for clinical severity, Pfaff's findings were confirmed, namely, that if caprinized rinderpest virus was administered 48 hours or more before virulent rinderpest virus the cattle were protected and underwent a caprinized virus reaction only. Neutralizing or complement-fixing antibodies have never been detected in cattle within 48 hours of vaccination (Plowright and Ferris, 1958) and consequently the protection can only be attributed to interference.

Our findings failed to support the belief that caprinized rinderpest virus possesses a therapeutic action in cattle already infected with virulent rinderpest virus. In fact, the simultaneous inoculation of both viruses and the inoculation of caprinized less than 33 hours before the virulent virus produced virulent rinderpest reactions. Unfortunately the virulent virus used in these experiments killed only 9·1 per cent of the cattle inoculated or 12·5 per cent of the cattle at risk and consequently the assessment of any therapeutic action could only be subjective and as such is open to criticism. Nevertheless the system adopted revealed highly significant differences between cattle given virulent virus only and cattle given caprinized virus only.

CONCLUSIONS

Cattle inoculated with virulent rinderpest virus before, simultaneously or up to 33 hours after caprinized rinderpest virus underwent a typical virulent rinderpest reaction. Cattle given virulent virus 48 and 57 hours after vaccination with caprinized rinderpest virus did not contract virulent rinderpest but underwent a typical vaccine virus reaction.

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TREATMENT OF RINDERPEST-INFECTED CATTLE WITH CAPRINIZED RINDERPEST VIRUS

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INTRODUCTION

Caprinized rinderpest virus is used widely in Africa as a live virus vaccine and field veterinarians are even advised to control actual outbreaks of the disease by vaccination. In practice both the healthy and the sick are vaccinated and many field veterinarians firmly believe that caprinized rinderpest virus has a therapeutic effect. Edwards (1950) likewise asserted that the vaccine virus was therapeutically active and he attributed its action to interference with the virulent virus. In West Africa, Marcque and Falley (1950) carried out trials with caprinized rinderpest virus in cattle infected with rinderpest. A total of 19 cattle were left untreated and all developed typical acute rinderpest and some died, whereas 42 cattle given caprinized virus underwent abortive reactions. On the other hand, the findings of Wilde and Scott (1961) failed to support the belief that caprinized rinderpest virus possessed a therapeutic action in cattle already infected with virulent rinderpest virus. Unfortunately, however, their strain of virulent rinderpest virus proved to be relatively avirulent and few cattle died. Accordingly their clinical assessments were of necessity arbitrary. The therapeutic effect of caprinized rinderpest virus was therefore reappraised in cattle infected with a highly virulent strain of rinderpest virus.

MATERIALS AND METHODS

Caprinized rinderpest virus. Aliquots of five different batches of known titres of the Kabete attenuated (KAG) strain of caprinized rinderpest virus were used. The virus was stored at minus 20°C as powdered freeze-dried infected goat spleens in vacuum-sealed ampoules. It was reconstituted prior to inoculation such that each 2·0 ml dose contained 3,000 cattle ID_{50}.

Virulent rinderpest virus. Aliquot suspensions were prepared from five different spleens from cattle infected with the Kabete "O" (RBKO) strain of virulent rinderpest virus. The infected spleens were stored whole at minus 20°C until required. The titre of each spleen was determined in cattle and suspensions were made immediately prior to inoculation such that each 2·0 ml dose contained 3,000 cattle ID_{50}.

Cattle. High grade crosses of various European breeds were used when two years old and weighing about 200 kg. They were purchased in the rinderpest-free areas of Kenya, and, on arrival, each was bled for serum which was examined for rinderpest neutralising antibodies. Only proven susceptible cattle were used.

Experimental design. Groups of 25 cattle were inoculated subcutaneously with 3,000 ID_{50} RBKO and a latin square design was used to test five

treatments, five cattle being given each treatment. A table of random numbers was used to assign the treatments to the cattle. The five treatments were: A = no KAG; B = KAG on the prepyrexial day; C = KAG on the second pyrexial day; D = KAG on the fourth pyrexial day and E = KAG on the sixth pyrexial day. RBKO from different infected spleens and KAG from different batches were used in the five replicate trials.

The inoculated cattle were housed for three weeks and then were held in convalescent paddocks for a further two weeks. Rectal temperatures were taken every morning and the cattle were examined daily. All cattle which died were autopsied and, where appropriate, the clinical and post-mortem findings were confirmed by the demonstration of specific rinderpest-diffusible precipitinogen in the lymph nodes.

Statistical analysis. The attributes measured were the case mortality rates and the reciprocals of the survival times. The data were subjected to the Analysis of Variance.

RESULTS

Morbidity Rate

Every inoculated animal developed a fever and clinical signs of rinderpest.

Case Mortality Rate

A total of 103 cattle died, giving a case mortality rate of 82·4 per cent. The distribution of deaths by treatment and replicate are shown in Table 1. Differences between treatments or between replicates were not significant ($P > 0{\cdot}20$ and $P > 0{\cdot}05$ respectively (Table 2).

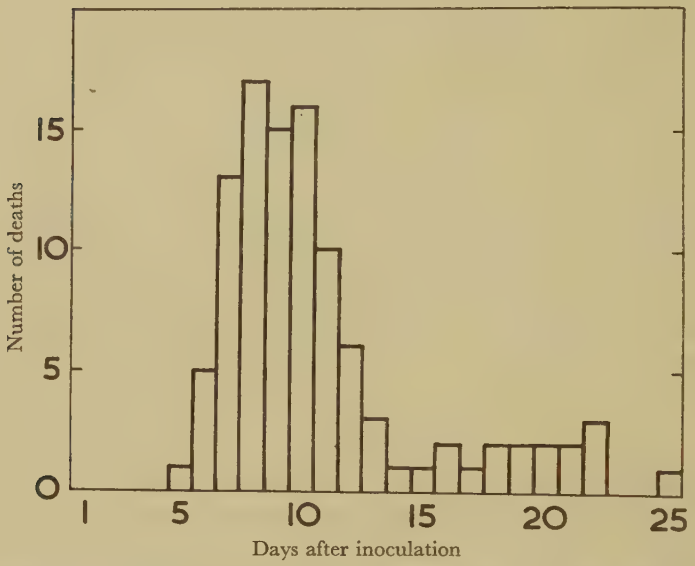
TABLE 1

DISTRIBUTION OF DEATHS IN THE GROUPS OF FIVE RINDERPEST-INFECTED CATTLE TREATED WITH CAPRINIZED RINDERPEST VIRUS

Replicates	*Treatments*					*Totals*
	A	*B*	*C*	*D*	*E*	
1	4	3	0	2	5	14
2	5	3	4	5	4	21
3	4	5	5	5	4	23
4	5	4	4	5	5	23
5	5	5	4	3	5	22
Totals	23	20	17	20	23	103

The histogram of the days of death was skewed (Fig. 1). The majority (83·5 per cent) of the cattle died before the 14th day after inoculation with RBKO. Accordingly a further analysis was made

Fig. 1



Histogram of the days of death of 103 rinderpest-infected cattle treated with caprinized rinderpest virus.

TABLE 2
ANALYSIS OF VARIANCE OF THE CASE MORTALITY RATES

Source	*Degrees of freedom*	*Sum of squares*	*Mean square*
Between treatments ..	4	5·04	1·26
Between columns ..	4	3·44	0·86
Between replicates ..	4	11·44	2·86
Error	12	14·72	1·23
Total	24	34·64	

of the data after omitting late deaths (Table 3). Again differences between treatments or between replicates were not significant ($P>0.20$ and $P>0.10$ respectively) (Table 4).

Survival Times

The reciprocals of the survival times were used because of the skewness of the days of death. Cattle which survived for five weeks were considered to have survived for infinite time for the purpose of calculating mean responses (Table 5). Differences between treatments or between replicates were not significant ($P>0.20$ and $P>0.10$ respectively) (Table 6).

TABLE 3

DISTRIBUTION OF EARLY DEATHS IN THE GROUPS OF FIVE RINDERPEST-INFECTED CATTLE TREATED WITH CAPRINIZED RINDERPEST VIRUS

Replicates	*Treatments*					*Totals*
	A	*B*	*C*	*D*	*E*	
1	3	1	0	2	5	11
2	3	2	4	4	3	16
3	3	4	4	5	4	20
4	5	3	4	4	5	21
5	5	4	3	2	4	18
Totals	19	14	15	17	21	86

TABLE 4

ANALYSIS OF VARIANCE OF THE CASE MORTALITY RATES OMITTING LATE DEATHS

Source	*Degrees of freedom*	*Sum of squares*	*Mean square*
Between treatments	4	6·56	1·64
Between columns	4	0·56	0·14
Between replicates	4	12·56	3·14
Error	12	20·48	1·71
Total	24	40·16	

TABLE 5

MEAN RECIPROCAL SURVIVAL TIMES (DAYS) OF GROUPS OF FIVE RINDERPEST-INFECTED CATTLE TREATED WITH CAPRINIZED RINDERPEST VIRUS

Replicates	*Treatments*				
	A	*B*	*C*	*D*	*E*
1	0·0960	0·0542	0·0000	0·0376	0·0954
2	0·0902	0·0578	0·0876	0·1104	0·0754
3	0·0848	0·0886	0·1186	0·1036	0·1092
4	0·1094	0·0862	0·0900	0·0960	0·1236
5	0·1252	0·1058	0·0766	0·0462	0·0982

TABLE 6

ANALYSIS OF VARIANCE OF THE MEAN RECIPROCAL SURVIVAL TIMES

Source	*Degrees of freedom*	*Sum of squares*	*Mean square*
Between treatments ..	4	0·00335931	0·00083983
Between columns ..	4	0·00133915	0·00033479
Between replicates ..	4	0·00666064	0·00166516
Error	12	0·00883744	0·00073645
Total	24	0·02019654	

DISCUSSION

The course of disease in cattle infected with a highly virulent strain of rinderpest was not influenced by the inoculation of caprinized rinderpest virus and our findings support the similar conclusion of Wilde and Scott (1961) who used a relatively avirulent strain to infect cattle.

The need for adequate controls in this type of therapeutic assessment was well illustrated in the first trial in which all five cattle given caprinized virus on the second pyrexial day survived. When the trial was replicated and 25 animals had undergone this treatment only eight survived, but to have been statistically significant ($P<0.05$) 12 cattle would have had to survive. Subsequently the first trial was repeated using aliquots of the same spleen to infect the cattle and aliquots of the same batch of caprinized virus for treatment. The distribution of deaths and the survival times simulated the average response of the latin square experiment.

CONCLUSION

The inoculation of caprinized rinderpest virus into cattle infected with a highly virulent strain of rinderpest virus failed to alter the course of the disease.

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INFECTION OF LABORATORY ANIMALS WITH *MYCOBACTERIUM JOHNEI*

IV. Comparative Susceptibility to Infection of C.57, C.B.A and Swiss White Mice

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INTRODUCTION

The results obtained by different workers on the susceptibility of mice to *Mycobacterium johnei* infection vary (Francis, 1943; Lominski, Cameron and Roberts, 1956; Ford, 1957; Chandler, 1961a). It has been suggested that differences between the various strains or breeds of mice may play a part. The following investigation, a note on which has been reported (Chandler, 1961b), was designed to evaluate any differences in susceptibility between three distinct strains of mice.

MATERIALS AND METHODS

Mice. Stocks of C.57 strain black mice and C.B.A. strain brindle mice were established for the purpose of this experiment from breeding pairs obtained from the Medical Research Council Radiobiological Unit, Harwell, by courtesy of Dr. M. F. Lyon. A stock of Swiss white mice has been maintained at the Agricultural Research Council Field Station, Compton, for several years. The mice were used when newly weaned at three or four weeks old. The management of the three strains at Compton was identical in all respects. The diet consisted of a basic mixture containing crushed wheat, oats, wheatings, bran, barleymeal, fishmeal, yeast, meat and bone meal and flaked maize; to this was added milk and bread, and cod liver oil was given once weekly.

Mycobacterium johnei. The strain of *M. johnei* was one originally isolated from a case of Johne's disease in a cow and was cultured on the medium of Taylor (1950). Six to seven week old growths of bacilli were suspended in saline and each mouse was inoculated intraperitoneally with 2 mg. of the moist bacilli in a volume of 0·25 ml.

Examinations. Liver and spleen impression smears, histological preparations and cultures were made and examined as already described (Chandler, 1961b). Faeces smears were made and examined in the usual manner. Intestinal smears were made by scraping the internal wall of an opened intestine and avoiding contact with the serosa as far as possible. Preparations for histological examination were made from sections cut transversly. Up to four separate pieces of intestine were taken from each intestinal area and mounted in one wax block for processing to a slide preparation containing four separate transverse sections; in this manner as many as 16 different intestinal sections were examined for each mouse. Staining throughout was by the Ziehl-Neelsen method and occasionally preparations stained by haematoxylin and eosin were examined; a preparation was classed as infected only if acid-fast stained bacilli were detected.

RESULTS

Experiment to Study the Course of Infection over a Period of One Year and Three Months

Fifty female mice of each strain were inoculated from the same source on the same occasion, and samples of mice were killed and examined at intervals viz: 3, 6, 9, 12 and 15 months after infection. During the course of the experiment 6 C·57 mice, 2 C.B.A. mice and 12 Swiss mice died. Death was diagnosed as due to Johne's disease of the intestines in 3 of the C·57 mice (dying 6 months, 12 months and 14 months after infection) and in 2 of the Swiss mice dying 10 months and 12 months after infection). Death in the other mice was due to various causes, e.g. neoplasms.

Three Month Sample

Three months after infection 15 mice of each strain were killed and examined.

Post-mortem appearance. The spleen size varied in the three strains of mice; C.57 spleens were all enlarged, C.B.A. spleens were very small and Swiss spleens were intermediate in size.

Smears and histology. The results of count made on liver and spleen smears and sections showed somewhat higher infections in the C.57 mice, when compared with the other two strains. One ileum and one colon section was examined from each mouse; 2 of the C.57 mice and one Swiss mouse, but none of the C.B.A. mice, showed intestinal infection.

Cultures. These were made of the liver and spleen of every mouse sampled. All but one sample, from a C.57 mouse liver which was contaminated, produced luxuriant growth, indicating the presence of viable bacilli.

Six Month Sample

Six months after infection 6 mice of each strain were killed and examined.

Post-mortem appearance. Spleen sizes varied in the three strains of mice as noted at three months.

Smears and histology. The results of counts made on liver and spleen smears showed no differences between the strains of mice. The results of histological examinations are summarised in Table 1. One sample of each portion of the intestine was taken, and two transverse sections (a) and (b) were prepared from each portion, the distance between each section being approx. 150μ. It will be seen from the Table that the Swiss livers showed the highest infections and the C.B.A. livers the least infection; spleen infections were highest in the C.57 mice. The intestinal examinations showed all of the C.57 mice, three of the Swiss mice, but none of the C.B.A. mice to be affected.

Cultures. Viable bacilli were present in the liver and spleen of every mouse sampled.

TABLE 1

RESULTS OF HISTOLOGICAL EXAMINATIONS MADE SIX MONTHS AFTER INFECTION

Breed	*Mouse*	*Liver*	*Spleen*	*Duodenum* (a)	*Duodenum* (b)	*Jejunum* (a)	*Jejunum* (b)	*Ileum* (a)	*Ileum* (b)	*Colon* (a)	*Colon* (b)	*Proportion of mice with intestinal infection*
C57	B41	1/8	1/4	++	+	−	−	+	−	−	−	6/6 = 100%
	B42	1/7	1/1	−	+	−	−	−	−	−	−	
	B43	1/20	1/3	−	−	−	−	+	+	−	−	
	B44	1/9	1/2	+	+	−	−	−	−	−	−	
	B51	1/3	2/1	−	−	+	+	+	+	−	−	
	B52	1/3	2/1	+	+	+	+	−	−	−	−	
CBA	R41	1/2	1/3	−	−	−	−	−	−	−	−	0/6 = 0%
	R42	1/30	1/4	−	−	−	−	−	−	−	−	
	R43	1/3	1/5	−	−	−	−	−	−	−	−	
	R44	1/20	1/30	−	−	−	−	−	−	−	−	
	R45	−	−	−	−	−	−	−	−	−	−	
	R51	1/4	1/10	−	−	−	−	−	−	−	−	
Swiss	W41	1/3	1/30	−	−	−	−	−	−	−	−	3/6 = 50%
	W42	1/4	1/10	−	−	−	−	−	−	−	−	
	W43	1/2	1/20	−	−	−	−	−	−	−	−	
	W44	1/2	1/2	−	+	+	+	−	−	+	+	
	W45	2/1	3/1	+++	+++	++	++	−	−	+	+	
	W51	1/2	1/10	−	+	−	−	−	−	−	−	

The fractions denoting intensity of infection in the liver and spleen represent the number of groups of bacilli per numbers of microscope fields. The symbols denoting intensity of infection in the intestines are as follows:—

+++ = Gross infection
++ = Numerous groups of bacilli
+ = Few groups of bacilli
− = No bacilli seen.

TABLE 2

RESULTS OF HISTOLOGICAL EXAMINATIONS MADE NINE MONTHS AFTER INFECTION

Breed	*Mouse*	*Liver*	*Spleen*	*Duodenum* (a)	(b)	(c)	(d)	*Jejunum* (a)	(b)	(c)	(d)	*Ileum* (a)	(b)	(c)	(d)	*Colon* (a)	(b)	(c)	(d)	*Proportion of mice with intestinal infection*
C57	B53	1/1	1/1	++	++	++	++	++	+++	+++	++	++	++	+	+	++	+	++	++	6/6=100 %
	B54	1/2	1/2	+	++	+	++	+++	+++	+++	+++	+++	++	+++	+++	++	++	++	++	
	B55	1/2	2/1	+++	+++	+++	+++	++	++	+++	+++	++	++	++	++	++	++	++	+	
	B61	1/2	2/1	+++	+++	++	++	++	++	++	+++	+	+	+	+	+	++	++	+++	
	B62	1/2	1/3	+	+	+	+	+	++	++	+	—	+	+	+	+	—	—	—	
	B63	2/1	5/1	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
CBA	R52	1/4	1/15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0/6=0%
	R53	1/40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	R54	1/5	1/10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	R55	1/5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	R61	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	R62	1/3	1/15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Swiss	W61	1/10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1/6=17%
	W62	—	1/15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	W63	1/30	1/3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	W71	1/30	1/15	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	
	W72	1/30	1/15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	W73	1/3	1/5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

For symbols—see Table 1

Nine Month Sample

Nine months after infection 6 mice of each strain were killed and examined.

Post-mortem appearance. The spleen sizes varied in the three strains of mice in the manner noted before. The intestines, in particular the duodenum and jejunum, of all the C.57 mice were visibly enlarged, due to thickening of the mucosa, and they were pale and more opaque.

Smears and histology. The results of counts made on the liver and spleen smears indicated a higher intensity of infection in the C.57 mice. Intestinal smears showed all the C.57 mice to be infected, and none of the C.B.A. mice. The results of histological examinations are summarised in Table 2. Four samples of each portion of the intestine were taken, and transverse sections (a, b, c, d) prepared from each portion. It will be seen from the Table that the C.57 livers and spleens showed the greatest degree of infection. The intestinal examinations showed all of the C.57 mice to be affected, high intensities of infection being recorded. Intestinal infection was noted in only one of the Swiss mice and in none of the C.B.A. mice.

Cultures. The livers and spleens of all the mice examined were shown to be infected with the exception of the liver in one Swiss mouse and one C.B.A. mouse, and the liver and spleen in a further mouse of each of these strains.

One Year Sample

One year after infection 6 mice of each strain were killed and examined.

Post-mortem appearance. The C.57 mice showed enlarged spleens, a lack of abdominal fat, hard nodular mesenteric lymph nodes and enlarged, pale, small intestines (see Figs. 1 and 2). The C.B.A. mice were much larger than the C.57 mice and showed marked abdominal fat, small spleens and apparently normal intestines. The Swiss mice were similar in size to the C.57 mice; one mouse (W.91) was emaciated and showed an enlarged spleen and enlarged small intestine, while the others showed smaller spleens.

Smears and histology. Intestinal smears demonstrated bacilli in 4 of the 6 C.57 mice (the lower infections not revealed in this manner were nevertheless shown histologically), in one of the Swiss mice (W.91), and in none of the C.B.A. mice. Faeces smears demonstrated bacilli in all the C.57 mice except one (B.10·5), in one Swiss mouse (W.91) and in none of the C.B.A. mice. The results of histological examinations are summarised in Table 3 from which it will be seen that the lowest level of liver infections occurred in the C.B.A. mice while the highest level of infection occurred in Swiss mouse W.91. The C.57 mice showed the highest level of spleen infections, with the exception of the individual Swiss mouse W.91. The intestinal sections showed infection of all the C.57 mice, one Swiss mouse (W.91) and a low grade infection in one C.B.A. mouse (R.61).

TABLE 3

RESULTS OF HISTOLOGICAL EXAMINATIONS MADE ONE YEAR AFTER INFECTION

Breed	*Mouse*	*Liver*	*Spleen*	*Duodenum* (a)	(b)	(c)	(d)	*Jejunum* (a)	(b)	(c)	(d)	*Ileum* (a)	(b)	(c)	(d)	*Colon* (a)	(b)	(c)	(d)	*Proportion of mice with intestinal infection*
C57	B101	1/10	1/2	+	+	+	+	++	++	++	+++	+	++	++	++	+++	+++	+++	++	6/6=100 %
	B102	—	1/2	—	+	—	—	+	—	+	++	—	—	—	•	—	+	—	+	
	B103	1/1	1/1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+++	++	+++	
	B104	1/1	2/1	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
	B105	1/10	1/2	—	+	—	+	+	+	+	•	—	—	—	—	—	—	+	•	
	B71	1/2	1/1	+	+	+	•	++	+	++	++	—	+	—	+	+	+	—	+	
CBA	R101	1/10	1/6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1/6=17%
	R102	1/10	1/8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	R103	1/20	1/10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	R104	1/20	1/7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	R61	1/6	1/5	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	
	R62	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Swiss	W91	3/1	3/1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1/6=17%
	W92	1/30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	W101	1/10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	W102	1/3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	W103	1/5	1/30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	W104	1/30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

For symbols—see Table 1

RESULTS OF HISTOLOGICAL EXAMINATIONS ONE YEAR AND THREE MONTHS AFTER INFECTION

Breed	*Mouse*	*Liver*	*Spleen*	*Duodenum* (a)	(b)	(c)	(d)	*Jejunum* (a)	(b)	(c)	(d)	*Ileum* (a)	(b)	(c)	(d)	*Colon* (a)	(b)	*Proportion of mice with intestinal infection*
C57	B71	1/1	2/1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	++	11/11=100%
	B72	1/1	3/1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
	B81	1/30	1/3	+	+	+	+	++	+	++	+	−	−	+	−	−	−	
	B82	1/1	2/1	+++	++	++	+++	+++	+++	++	+++	+++	++	+++	+++	+++	+++	
	B83	1/20	1/8	−	−	−	−	+	+	+	−	−	−	−	−	−	−	
	B84	1/15	1/1	+	+	+	−	++	++	++	++	+	++	++	+	+	++	
	B85	1/1	6/1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	
	B91	1/7	1/1	++	++	++	++	++	++	++	++	++	+	+	++	++	+++	
	B92	1/50	1/5	−	−	−	−	−	−	−	+	−	−	−	−	−	−	
	B93	1/6	1/2	+	+	+	+	+	−	+	−	−	−	−	−	−	−	
	B94	1/5	1/3	+	+	+	+	++	++	++	+	++	+	++	++	++	+++	
CBA	R71	1/3	1/4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/15=0%
	R72	1/6	1/2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R73	1/3	1/5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R74	1/5	1/5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R75	1/10	1/7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R81	1/4	1/10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R82	1/30	1/30	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R83	1/10	1/15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R84	1/20	1/6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R85	1/30	—	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R91	1/7	1/30	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R92	1/6	1/30	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R93	1/8	1/5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R94	1/10	1/15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	R95	1/10	1/10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
Swiss	W51	1/25	1/25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	1/5=20%
	W52	1/8	1/7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	W71	1/6	1/1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	
	W81	1/10	1/50	−	−	−	−	−	−	−	−	−	−	−	−	−	+	
	W82	1/8	1/3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	

For symbols—see Table 1

Cultures of livers and spleens revealed viable bacilli in all the C.57 mice with the exception of one spleen, in the livers of 3 C.B.A. mice and spleens of all C.B.A. mice, and the livers and spleens of 4 of the Swiss mice.

Termination of Experiment

One year and three months after infection the experiment was terminated by killing and examining all the surviving mice, i.e. 11 C.57 mice, 15 C.B.A. mice and 5 Swiss mice.

Post-mortem appearance. The majority of the C.57 mice were small, in particular number B.85. The majority of the C.B.A. mice were large, while the size of the Swiss mice was intermediate. The sizes of the spleens of the three strains were, in the majority of cases, as recorded at previous samplings. The intestines of 6 of the 11 C.57 mice were pale and enlarged; the remainder were normal in appearance. The intestines of all the C.B.A. mice appeared normal. The intestine of one of the Swiss mice (not positive on histological examination) appeared enlarged, while the remainder appeared normal.

Histology. The results of the histological examinations are summarised in Table 4. The greatest intensities of liver and spleen infections occurred in the C.57 mice; the infections in the spleens of the latter mice were in all cases of greater intensity than in the corresponding livers. Intestinal infection was seen in all of the C.57 mice; one mouse (B.85) showed the heaviest intestinal and spleen infection yet encountered (see Fig. 3). However, one mouse (B.83) showed only small groups of bacilli in the jejunum and one mouse (B.92) showed only one small group of bacilli, in one of the jejunum sections. None of the C.B.A. mice showed intestinal infection, and only one of the Swiss mice.

Pathology

Throughout this experiment the histological appearance of the lesions in livers, spleens and intestines was similar to that described earlier (Chandler 1961b). In mice showing intestinal infection, infection of the pancreas was in many cases also encountered (Fig. 4); the interstitial tissue was invaded with bacilli but the acinar cells and, as far as could be ascertained, the islets of Langerhans, were unaffected. The use of the intraperitoneal route may have resulted in this feature of infection. It was noted that in some of the cases of intestinal infection of low intensity, groups of bacilli were seen comparatively close to the junction of the mesentery with the intestine; in a section of jejunum of a mouse killed 9 months after infection, the serosa contained groups of bacilli and a series of small groups of bacilli were seen leading from the mesentery into the submucosa. These observations suggest that the route of infection of the intestine in these experimental cases may be from the serosa, via the mesentery, into the submucosa.

Course of Infection over a Period of Six Months

Since the major experiment had shown an interesting comparative result at the six month sample, a further 12 mice of each of the three strains were inoculated with *M. johnei* as before, using a different subinoculation of the same strain. All the mice were killed and examined six months later.

Post-mortem appearance. The C.B.A. mice were fatter and larger than the other two mouse strains, which nevertheless appeared in good condition. The C.57 mouse spleens were enlarged in comparison with those of the other strains.

Histology. The intensities of infection in the livers showed little difference between the three strains of mice; no bacilli were seen in the livers of 4 C.57 mice, 5 C.B.A. mice and 6 Swiss mice. It was evident that the general level of infection in all three strains of mice was low. Spleen examinations, however, showed a markedly higher intensity of infection in the C.57 mice, all the sections being positive; no bacilli were seen in the spleens of 9 of the C.B.A. and 6 of the Swiss mice. Four sections were examined from the duodenum, jejunum and ileum, and two sections from the colon. Four of the C.57 mice showed intestinal infection; no intestinal infection was seen in any of the C.B.A. or Swiss mice. Although the intestinal infections were in general of lower intensity than in the major experiment at this period, the greater susceptibility of the C.57 was nevertheless indicated.

DISCUSSION

The major experiment showed that the C.57 mice were more susceptible to *M. johnei* infection than the Swiss white mice, and the C.B.A. mice appeared least susceptible. The difference in susceptibility was shown in liver examinations and even more so in spleen examinations, but the most striking differences were in respect of intestinal infection. Intestinal infection was detected histologically in all the C.57 mice sampled six months after infection, and macroscopic lesions were seen post-mortem in all the C.57 mice sampled nine months after infection. In subsequent samples all the C.57 mice showed intestinal infection. Heavy infections were encountered, all parts of the intestine being affected; the small intestine was regularly affected and there appeared little advantage in additional examination of the colon.

Intestinal infections in the Swiss mice emphasised the differences in susceptibility in individual mice of this strain. Although heavy infections were encountered, in the most favourable sample only 50 per cent of these mice showed intestinal infection. The C.B.A. mice were in general resistant to intestinal infection, only one mouse showing infection in this site throughout the experiment. Only a small number of mice died showing intestinal infection during the experiment, though this might have been greater had all the mice been kept for the full period.

Infection of the interstitial tissue of the pancreas was recorded, and there was evidence suggesting that with intraperitoneal inoculation the route of infection of the intestine is from the serosa, via the mesentery into the submucosa.

The second experiment, in spite of the general low level of infection in all three strains, again indicated the greater susceptibility to infection of the C.57 mice.

The differences in susceptibility to infection shown by the three strains of mice kept in an identical environment suggest that genetic constitution may play a part in susceptibility to Johne's disease. C.57 mice show promise of being useful experimental laboratory animals for Johne's disease studies. Further experiments are in progress on these lines.

CONCLUSIONS

Groups of C.57, C.B.A. and Swiss White mice were inoculated intraperitoneally with *M. johnei* and the course of infection studied. The C.57 mice were more susceptible than the Swiss White Mice, and the C.B.A. mice were least susceptible.

Intestinal infection was detected with regularity in the C.57 mice sampled 6 months or more after infection, and macroscopic lesions were seen post-mortem; intestinal infection was detected in a minority of the Swiss White mice and in only one case in the C.B.A. mice.

The differences in susceptibility of the three strains of mice suggest that genetic constitution may play a part in susceptibility to Johne's disease. C.57 mice offer promise as experimental laboratory animals for Johne's disease studies.

ACKNOWLEDGMENTS

I wish to thank Mr. F. Summerfield for production of the photographs Mr. W. Moss for the breeding and management of the mice, and Mr. G. Jones and Miss J. Warne for technical assistance.

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Fig. 1. C.57 black mouse (right) and C.B.A. brindle mouse (left) one year after infection. The smaller size of the C.57 mouse, its enlarged spleen, opaque small intestine and lack of abdominal fat are shown.

Fig. 2. As in Fig. 1, but with viscera displayed. The enlarged spleen and thickened small intestine of the C.57 mouse (left) are demonstrated.

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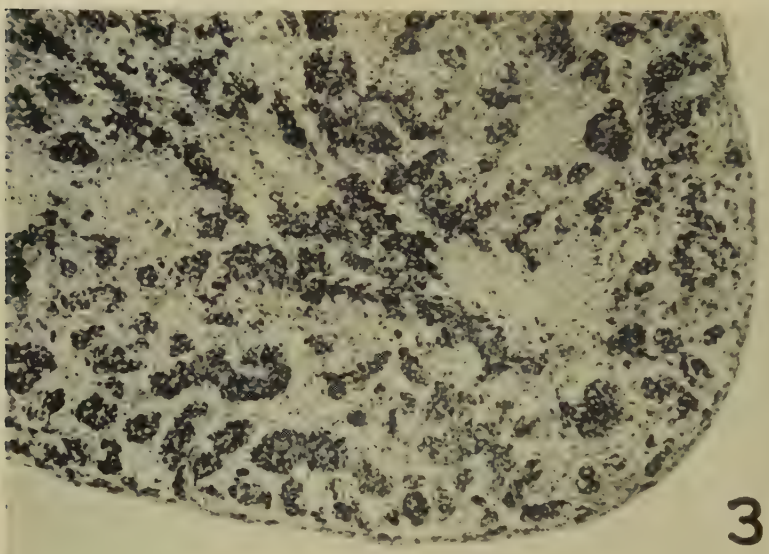

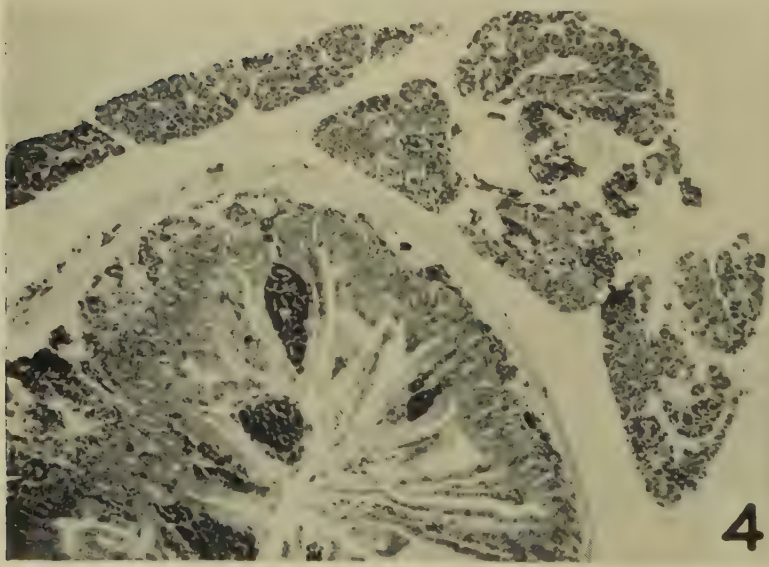

Fig. 3. Spleen of C.57 mouse (number B.85) showing gross infection. Stained by Ziehl Neelsen method; the darker groups of material are masses of acid-fast bacilli.

Fig. 4. Pancreas and part of the duodenum of C.57 mouse (number B.85). Stained b Ziehl-Neelsen method; the darker groups of material are masses of acid-fas bacilli.

EFFECT OF STREPTOMYCIN ON THE GROWTH RATE AND INTESTINAL FLORA (*ESCHERICHIA COLI*) OF PIGLETS

By

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INTRODUCTION

According to a recent survey by the Veterinary Investigation Service (1959), nearly 26 per cent of all piglets born fail to survive until they are eight weeks old. Up to three weeks of age, 13·8 per cent of the mortality is due to enteritis which also accounts for a similar proportion of the losses up to weaning age. Goodwin (1957) considers that enteritis is one of the commonest diseases of suckling pigs, and that it is responsible for deaths and a temporary check in their growth. The clinical syndrome recently described by Saunders, Stevens, Spence and Sojka (1960) includes the occurrence of diarrhoea and rapid death of piglets within 48 hours of birth as the more prominent features.

For a considerable time it has been thought that there is an association between *Escherichia coli* and the cause of the disease and Saunders *et al.* while investigating 58 outbreaks of enteritis in piglets, isolated two main serological types, E.68 and G.7, from piglets which had died of the disease. These two sero-types also accounted for the majority of the typable strains recovered by Sojka, Lloyd and Sweeney (1960) from piglets that had died of gastro-enteritis, and these authors consider that these organisms are pathogenic, although there may be predisposing factors to infection. According to the survey referred to earlier, it is evident that *E. coli* is the most common organism found in bacterial infections responsible for death in early life, so that it may be accountable for other forms of disease resulting in unthriftiness in the growing pig.

At Compton, where a herd of pedigree Large White pigs is maintained, a form of enteritis similar to that described has been observed. The main characters are the appearance soon after birth of a bright yellow diarrhoea, sometimes in all the piglets of a litter, lasting usually for 48 hours. Piglets severely affected rapidly lose condition, and about 5 per cent of the litters of sows may die, while in the litters of gilts the mortality may be much higher. At about three weeks of age, a second attack of diarrhoea usually occurs and this time the faeces are greyish-white in colour and the piglets may scour for a week. This secondary diarrhoea is more prevalent in the winter than in the summer months. While the death rate accounted for by these forms of enteritis may not be high, they are responsible for unthriftiness and a consequent reduction in body weight at the time of weaning.

A preliminary bacteriological examination of the faeces of affected and normal piglets in the herd showed the presence of a flora in which *E. coli* colonies predominated; these were, in the main, sensitive to streptomycin and chloramphenicol, but resistant to the tetra-

cylines. It was, therefore, decided to test the therapeutic value of administering streptomycin to a number of litters and also to study the effect of treatment on the *E. coli* microflora of the faeces. Streptomycin was chosen because it is known that this antibiotic is not readily absorbed but is excreted unchanged in the faeces, whereas chloramphenicol is rapidly absorbed from the intestinal tract and is excreted in the urine.

Under normal conditions of management where piglets and the sow occupy a large pen it is difficult to inspect each piglet closely or to judge the effect of treatment on the character of the faeces. It was decided therefore to assess any beneficial effect by making a comparison of the growth rate of treated and untreated piglets in the same litter.

MATERIALS AND METHODS

Piglets and their management. The experiments were carried out on piglets born and reared at the pig farm at Compton. Sows, after farrowing in an indoor pen, were transferred with their litters to moveable folds when they were one week old. From 10 days old, the piglets were offered a little creep feed supplemented with vitamins and this was replaced at 5 weeks by a weaner mixture made into pellets. The litters were weaned at 8 weeks, at which time they were brought indoors for store or fattening purposes.

Plan of experiments. The first experiment included eight litters; one half of each litter was treated with streptomycin from birth until four weeks old while the other half served as controls. The treated piglets in four of the litters were given antibiotic by mouth and those in the other four litters intramuscularly.

In the second experiment, streptomycin was given by mouth for one week only, beginning when the piglets were three weeks old, at a time when they might be affected by a second attack of scouring especially in the winter months. It involved two separate trials, one including 6 litters treated during winter, and the second the same number of litters treated in the following spring. Consecutive litters born within a convenient time were selected for each experiment. The piglets were weighed at birth and every week thereafter until they were weaned at 8 weeks.

Dose of streptomycin. The dose selected was 25 mg. which represented a daily rate of 25 mg./kg. at birth and 5 mg./kg. at 3 weeks when the piglets averaged about 11 lb. live weight. For oral administration, the dose was given as streptomycin sulphate included in a capsule or pill and, for intramuscular injection, as dihydrostreptomycin dissolved in 1 ml. of sterile distilled water.

Sensitivity of faecal E. coli. At birth and in each succeeding week, faeces samples taken by rectal swab were examined from the same two control piglets and two treated piglets selected from each litter. The swab was rubbed gently on the surface of MacConkey medium in a plate and two "Sentests"* tablets, one containing streptomycin and the other aureomycin, were placed on the medium. The susceptibility of the faecal flora was assessed by observing the zone of inhibition around the tablet.

From the plate prepared from each piglet, 3 to 5 colonies of *E. coli* were picked and their exact sensitivity determined in broth containing 2·5, 10,

* Prepared by Evans Medical Ltd.

50 and 100 μg./ml. streptomycin. One drop of an 18-hour broth culture of each colony was added to tubes containing 1 ml. of medium and, after overnight incubation, the sensitivity of the strain was established by the lowest concentration which inhibited growth completely when judged by visual examination. It was found that sensitive strains of *E. coli* were usually inhibited by a concentration of 2 μg./ml. and that resistant strains grew in the presence of 100 μg./ml.

Streptomycin in the faeces. Samples of faeces were examined for the presence of streptomycin. The technique employed was to prepare a phosphate-buffer extract (1 to 10) of the faeces and to test its power to inhibit Klebsiella 41 in a solid medium. The extract was placed within cylindrical holes punched out of the medium and the concentric zones of inhibition developing on incubation at 30°C. were measured and compared with those of standard dilutions.

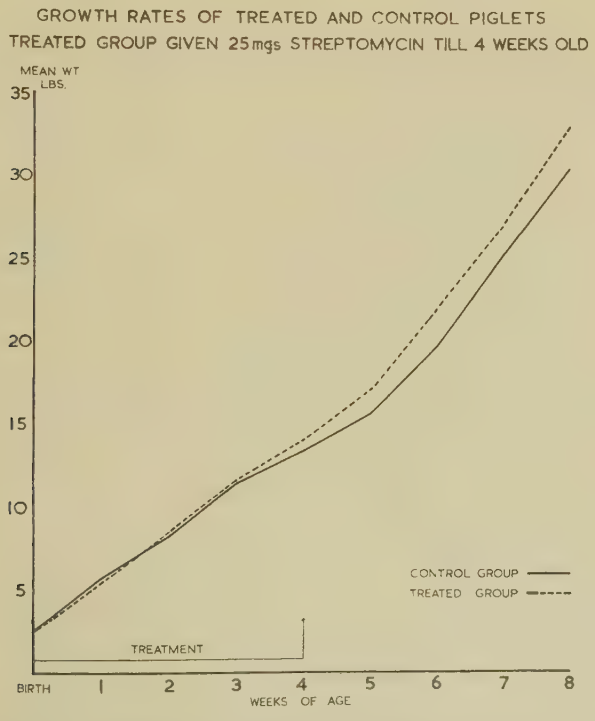
RESULTS

Treatment for Four Weeks

Growth Rate

Curves showing the growth rate of the controls and of the treated piglets in the eight litters are given in Fig. 1. while the average birth

Fig. 1



average weaning weight of 33 control piglets was 32 lb. and of 34 treated piglets 34·70 lb. representing an increased live weight gain and weaning weights of individual litters are seen in Table 1. The

TABLE 1

BIRTH AND WEANING WEIGHTS OF CONTROL AND TREATED PIGLETS

Litter	*1*		*2*		*3*		*4*		*5*		*6*		*7*		*8*	
	Control	*Treated O*	*Control*	*Treated I.M.*	*Control*	*Treated O*	*Control*	*Treated I.M.*	*Control*	*Treated O*	*Control*	*Treated I.M.*	*Control*	*Treated O*	*Control*	*Treated I.M.*
No. of piglets	4	4	3	4	3	3	4	3	5	5	4	5	5	5	5	5
Wt. at birth (lbs.)	2·1	2·25	2·3	2·35	2·8	2·6	2·52	2·39	3·42	3·19	2·35	2·65	2·05	2·35	3·19	3·01
Wt. at weaning—8 wks. lbs.	30·25	36·0	28·0	32·0	32·33	35·33	26·50	32·66	39·40	36·80	26·75	30·80	34·20	39·0	38·90	35·05
Average daily gain (lbs.)	0·503	0·603	0·459	0·529	0·527	0·584	0·428	0·540	0·643	0·60	0·435	0·502	0·574	0·654	0·64	0·572
Difference		+0·1		+0·07		+0·057		+0·112		—0·04		+0·067		+0·08		—0.068

Litters (O) given 25 mg. streptomycin daily by mouth
(I.M.) „ „ intramuscularly.

in the latter of 8·3 per cent. In Table 1, it will be seen that, with the exception of litters 5 and 8, there was an improved daily gain in weight in the treated piglets over that of the controls. In the six litters responding to treatment, the mean daily gain of the control piglets was 0·48 lb. and that of the treated piglets, 0·568 lb. with a mean difference of 0·08 ± 0·03 lb. The two litters which failed to respond belonged to sows which gave birth to strong piglets which did not scour. They all survived till weaning, whereas the other litters were lighter at birth and were affected with diarrhoea persisting up to four days; in the latter there had also been a mortality of 25 per cent in the piglets due to a variety of causes occurring at or soon after birth. It will be noticed that themaximum increase in daily gain was obtained in litter 4 in which diarrhoea was observed for 14 days. The average gain in weight over controls by piglets treated by the oral or by the intramuscular routes was the same.

Effect on Sensitivity of E. coli *from Faeces*

Plate test. The plate test on MacConkey agar showed that the faeces of piglets from all litters contained very large numbers of *E. coli* colonies. At birth, these colonies appeared to be mainly sensitive to streptomycin and resistant to aureomycin according to the definition given by Williams Smith and Crabb (1957). This status mainly persisted throughout the period of eight weeks in the case of control piglets. On the other hand, one week after commencing treatment, the organisms in the faeces of treated piglets became resistant to streptomycin and remained so during the four weeks of treatment. Two weeks after terminating treatment, the sensitivity of the organisms was restored to that existing at birth.

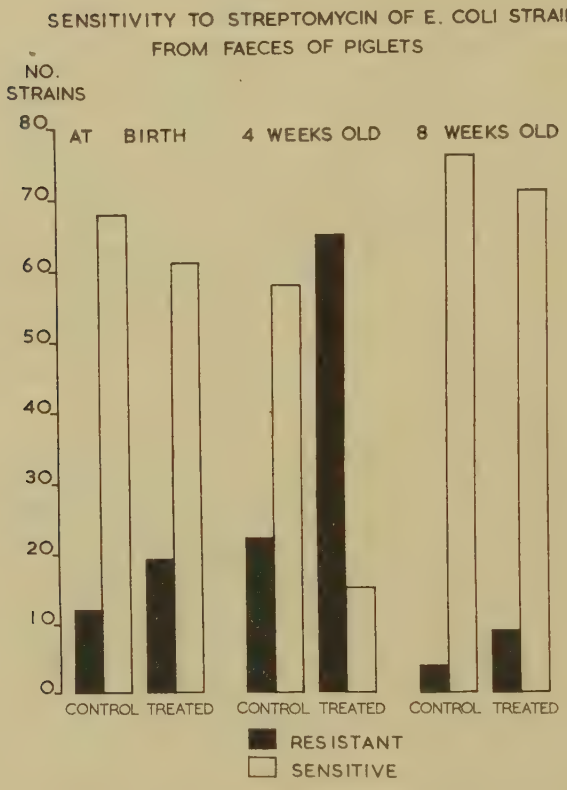
Colony sensitivity. Tests on individual colonies showed the presence of two varieties of *E. coli*, one being sensitive to streptomycin and one resistant. The effect of administering the antibiotic was to suppress the sensitive variety, at least for the duration of treatment.

The changes in the relative proportion of the two varieties at tests carried out at birth, four weeks, and eight weeks are shown in Fig. 2. At birth, about 80 per cent of all *E. coli* colonies examined were sensitive to streptomycin and the remainder resistant. After treatment with streptomycin for four weeks, the number of resistant organisms in the treated piglets had increased so that 80 per cent were resistant. At the test carried out when the piglets were eight weeks old, the organisms were almost entirely sensitive again. It will be seen in Fig. 2 that there was also an increase in the number of resistant organisms recovered from control piglets at four weeks. This suggests that resistant strains were acquired by the control piglets by infection from contaminated surroundings which were shared with the treated piglets.

Treatment for One Week

The previous experiment showed that the growth rate of piglets

Fig. 2

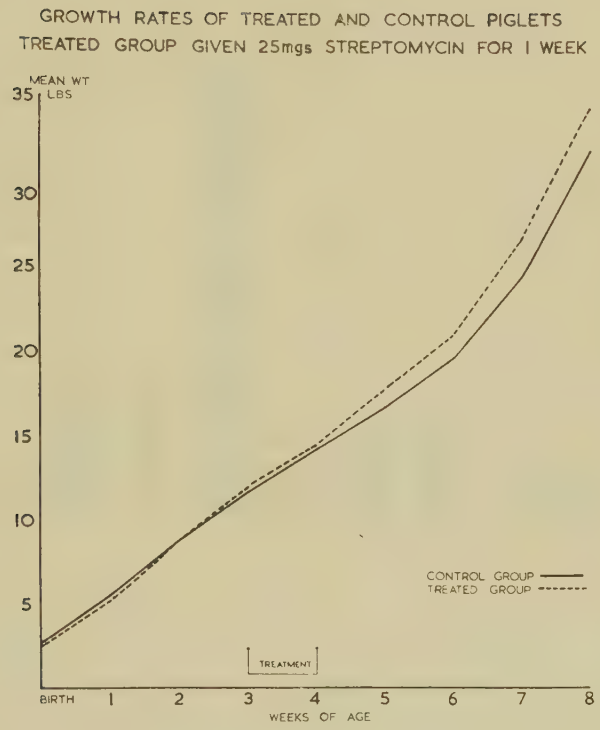


could be stimulated by giving streptomycin for four weeks, but that the organisms in the intestinal tract, notably *E. coli*, had developed a resistance to the antibiotic within this period. It was therefore decided to study the effect produced on the growth rate and on the rapidity with which changes in the sensitivity of *E. coli* developed when antibiotic was given for one week only.

Growth Rate

The mean weekly growth weights of 22 control and 26 treated piglets of the six litters in the first trial are shown in Fig. 3. The sows had been included in the last experiment and the growth response of the treated piglets in the present experiment was similar to that seen in their last litters. The treated piglets of five out of the six litters showed a gain in daily weight over that of the control piglets—0·56 lb.—compared with 0·49 lb. with a mean difference of 0·06 ± ·03 lb. In the 6th litter however, the treated piglets showed a decrease as they had done in the last experiment when the sow had reared a very good litter.

Fig. 3

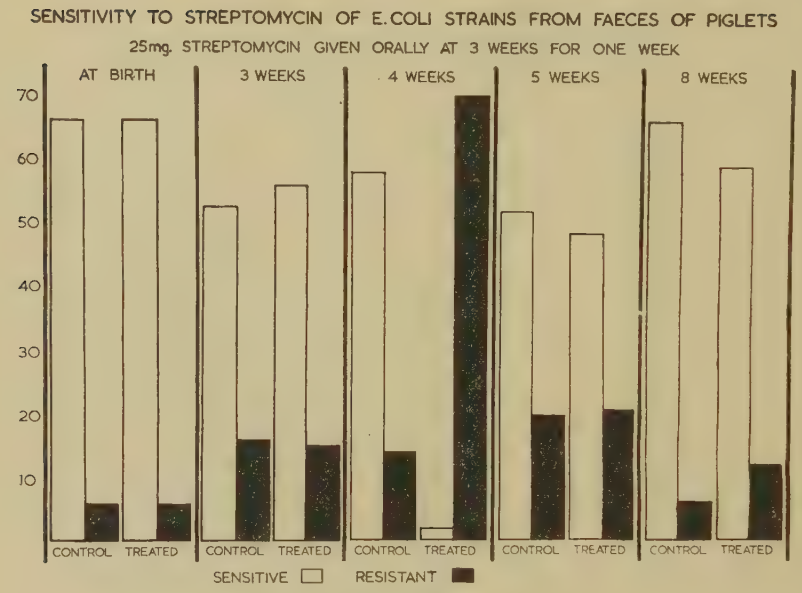


In the second trial, one litter was omitted from the results because of the reduction in its number by three deaths in the control piglets. While the treated piglets in each of the remaining five litters all showed gains in their final weight and in their daily gain over the controls, the increases were not as great as in the first trial carried out in the preceding winter. On the other hand, the average daily gain in both the control and treated piglets was greater in this group and the incidence of diarrhoea occurring at three weeks was lower.

Effect on E. coli *Sensitivity*

The results of successive tests on the 12 litters included in the two trials are shown in Fig. 4. It was found that, before treatment, the majority (92 per cent) of the colonies of *E. coli* examined were sensitive to streptomycin. At the test carried out when the piglets were four weeks old and had received treatment for one week, 70 out of 72 colonies (97 per cent) recovered from treated piglets were resistant to streptomycin. One week after treatment was discontinued, however, the original proportion of sensitive and resistant strains was being re-established, while at weaning time at 8 weeks, the flora was again mainly sensitive.

Fig. 4



DISCUSSION

These experiments show that the administration of streptomycin to piglets improves their growth rate. It has been found that the weaning weights of the pigs in the herd where it was tried tend to be below normal and a high incidence of piglet diarrhoea is believed to be responsible for the depression in growth. The therapeutic value of antibiotics in this condition has been shown by Saunders *et al.* (1960) who used streptomycin orally, and by Schipper, Buchanan and Eveleth (1956) who injected terramycin into the sow.

Investigations into the use of antibiotics in pigs during the fattening stage have in the main showed the advantage of penicillin and aureomycin in improving their growth rate and food conversion (Rpt. Agricultural Research Council, 1953; Gordon and Taylor, 1953), but the limited trials carried out on suckling pigs in this country have given variable results. The present investigation also shows that there is a variation in the growth response of individual litters when streptomycin is administered from birth to four weeks of age and that there is no advantage for vigorous litters of good birth weight. In two comparable trials carried out in winter and in summer, where streptomycin was only given for one week, the responses were greater in the winter period when scouring at three weeks was more prevalent.

More significant than the effects on growth rates are the effects on the *E. coli* flora of the faeces. In the first place they confirm those of Williams Smith and Crabb (1957) who found in the case of the tetracyclines that a predominantly resistant population of organ-

isms develops in the intestine. An examination of individual colonies however, has shown that before treatment with streptomycin, a sensitive as well as a resistant variety of *E. coli* is present in the faeces and that treatment with this antibiotic has the effect of altering the proportion by selective inhibition of the former variety; once the antibiotic has been withheld the original proportion is re-established.

Observations on the growth-promoting effect of streptomycin suggest that there is no advantage to be gained in administering it beyond the point at which the population of organisms has become mainly resistant to its action. It would be reasonable to suppose therefore that a form of interrupted administration would be worth a trial, the antibiotic being given for a short period followed by one in which it was withheld and during which a sensitive population was allowed to develop. This procedure takes into account the view that the suppression of *E. coli* present in the intestine of the piglet is of special advantage, but support could only be given to the validity of this assumption if it could be shown that the serotypes now believed to have pathogenic importance were also sensitive to streptomycin.

CONCLUSIONS

The administration of streptomycin in a dose of 25 mg. daily to piglets in 8 litters from birth until four weeks of age resulted in a live weight gain of 8 per cent over that of untreated piglets at the time of weaning at 8 weeks. The best average daily gain was obtained in litters having a poor birth weight and affected with piglet diarrhoea.

A similar growth response was obtained when the antibiotic was given for one week only at 3 weeks of age to piglets in six litters farrowed in the winter. A smaller response was obtained in five litters which were treated in the spring and were not affected with diarrhoea to the same extent.

Examination of the streptomycin sensitivity of *E. coli* organisms present in the faeces showed that a predominantly sensitive flora was replaced by a resistant flora within one week of commencing treatment. After treatment was discontinued the original proportion of sensitive and resistant organisms was restored.

ACKNOWLEDGMENT

I wish to express my thanks to Glaxo Laboratories Ltd. who supplied the streptomycin in suitable form for giving to piglets.

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THE HISTOPATHOLOGY OF THE EXTERNAL AUDITORY MEATUS OF THE DOG

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INTRODUCTION

Although external otitis is one of the most common conditions affecting dogs there are few references in the literature to the normal and pathological histology of the external acoustic meatus.

While it is generally agreed that the description of the histology of the human auditory meatus applies equally to that of animals, Trautmann and Fiebiger (1952) have shown that in dogs, and other carnivores, hairs and ceruminous glands occur throughout the external auditory canal and that sebaceous follicles are especially prominent in the osseous parts. Neilsen (1953) is of the opinion that the apocrine and sebaceous glands in the ear are of a similar structure to those of the skin and that the normal ear secretion, the cerumen, is a product of both glandular types. He also observed marked hypertrophy and proliferation of the sebaceous and apocrine glands in a number of affected ears. Witter (1949), on the other hand, considers that a type of ceruminous gland occurs only in diseased canine ears as the result of metaplastic changes and is probably responsible for the reddish-brown exudate which is a feature of most cases of chronic external otitis.

In view of the great dearth of information regarding the tissue changes in the external ears of dogs suffering from the various clinical forms of otitis, it was decided to examine a number of affected ears histologically and to compare the findings with those of healthy dogs.

METHODS

During a recent study of canine external otitis (Fraser, 1957), a number of dogs examined were subsequently destroyed for various reasons such as old age and concurrent disease or because they failed to respond satisfactorily to a prolonged course of treatment. All were examined post-mortem and from 20 dogs known to be suffering from otitis at the time of death, 27 external ears were removed for histological examination. In addition, material was obtained from the ears of 10 clinically normal dogs, which were destroyed at the owners' request for reasons other than disease e.g. "biting", "moving house", etc., care being taken to ensure that the external ear canals showed no visible abnormality.

Small pieces of tissue were removed from the external ears, at three levels, and were placed in 10 per cent formol-saline. Sections were prepared by standard methods and stained with haematoxylin-eosin, by Gram's method and by Gomori's aldehyde fuchsin staining technique (Gomori, 1950).

Material from the external ear canals of healthy and affected dogs was also examined bacteriologically and an attempt was made to correlate the findings with the tissue changes present.

RESULTS

Healthy Ears

The large conchal cartilage was invested on both sides with skin, the structure of which was typical of that of the rest of the body except that in most breeds the concave inner surface contained fewer hair follicles than on the outside. The most superficial skin structure, the epidermis, was thinner than the underlying corium and consisted of four or five distinct layers of cells. The corium, or dermis, was in two parts, the more superficial consisting of a mixture of delicate reticular fibro and fibro-elastic tissue, the rest being an interwoven network of collagenous and elastic fibres. On the other hand, the hypodermis or subcutis was of a much looser meshwork of elastic fibres and contained considerable amounts of adipose material with numerous large sebaceous glands and, in the deeper layers, a few tiny tubular structures resembling ceruminous glands.

Histologically, the auditory canal closely resembled the external ear or pinna except that, in most breeds, hairs were fewer and did not extend throughout the external acoustic meatus. Large, actively secreting sebaceous follicles were again very prominent and were especially numerous in the more distal sections (Figs. 1 and 2). These multiple, branched alveolar glands, with their holocrine type of secretion, invariably opened by means of a short common duct into the associated hair follicle a short distance below the skin surface, whereas the tubular ceruminous glands, which closely resembled the apocrine glands of human ears, were mostly situated in the deeper dermal layers below the corresponding sebaceous glands. The secretory portion of a ceruminous gland was usually lined with high columnar cells in contrast to the tubular part which was mostly lined with a single row of flattened, elongated cells, with faintly staining nuclei.

Changes in the External Auditory Canals of Dogs Suffering from Otitis

The epidermis. The majority of affected ears showed early hyperplastic changes of the epidermis including hyperkeratinization of the hair follicles, generalised thickening of the epithelium to five or six times its normal thickness (Fig. 3) with, or without, the formation of distinct "rete-pegs" extending between the dermal papillae (Fig. 4). Also frequently seen were dense aggregations of inflammatory cells, in the region of the prickle cell layer, and varying degrees of hyperplasia of the retemucosum. Hyperaemia and ulceration of the lining epithelium were frequently observed in chronically affected ears, especially those infected with *Pseudomonas* or *Proteus* bacilli.

Fibrous tissue. Fibroplasia was also a feature of most longstanding cases of external otitis and frequently resulted in thickening of the subcutaneous tissues with partial occlusion of the external auditory canal (Fig. 5).

Glandular tissue changes. Compared with the large, actively secreting sebaceous glands of clinically normal ears, the sebaceous follicles

in chronically affected ears were usually smaller and less active and tended to be displaced in the superficial dermal layers by the grossly dilated ducts of the ceruminous or tubular apocrine glands (Figs. 6, 7 and 8). Although the sebaceous glands appeared as evaginations of the hair follicles and their several follicles united into a common group, with a single opening well below the surface of the epidermis, the modified ceruminous glands reached the free surface of the skin either immediately adjacent to the opening of the associated hair follicle or at some distance from it (Figs. 9 and 10). In healthy ears and in many of the earlier, uncomplicated cases of otitis, fine straight tubular ducts were difficult to distinguish, whereas in chronically affected ears they usually occurred as large saclike diverticula distended with eosinophilic, homogeneous, colloidal material (Fig. 8). These large cystic structures, which almost completely displaced the less active sebaceous glands in the superficial layers of the hypodermis, were usually associated with *Proteus* and *Pseudomonas* infection, in which the secretions were copious and of a purulent nature.

Tumours. Two types of tumour were encountered during the survey. The first was an adenocarcinoma of the ceruminous glands which showed numerous glandular cavities of various shapes and sizes containing cellular debris, inflammatory cells and large plaques of colloidal material. The rest of the corium consisted of dense areas of fibrous tissue with apparently normal ceruminous-type glands throughout. At one point the hyperplastic glandular material was seen to extrude beyond the annular cartilage. The lining membrane of the grossly distended glands gave rise to papillary projections (Fig. 11) which closely resembled the pattern seen in an intraductal carcinoma of the mammary gland. The associated parotid lymph node showed marked lymphoid hyperplasia without evidence of metastasis.

The second type of tumour involved the sebaceous glands and was characterised by the presence of numerous pockets of hyperplastic glandular material infiltrating the connective tissue of the hypodermis (Fig. 12). Although there was marked hyperkeratinisation of the epithelium and thickening of the rete mucosum, there was no evidence of rete-peg formation, ulceration, or dilation of the tubular apocrine glands.

Tissue Mast cells. Several workers, including Larrson (1957), have reported on the high mast cell content of canine tissues. Although adult tissue mast cells were comparatively rare in the healthy external ear canal, they were usually fairly numerous in the skin overlying the auricular cartilage and the more distal parts of the conchal cartilage. In otitis, however, their presence in the connective tissues seemed to depend on the severity of the clinical condition. In the early stages of the disease only a few mast cells were seen scattered throughout the superficial dermal layers, whereas in chronically affected ears, especially those infected with Gram-negative pyogenic bacteria, they were present in large numbers in areas of marked

inflammatory reaction. Most of the cells in this region were irregular in shape and contained numerous metachromatic granules, whereas in healthy ears and in those affected with the acute form of otitis many of the mast cells showed vaculation of the cytoplasm with a tendency to disruption.

The Bacterial and Fungal Flora

Material from affected external ears was examined to determine the possible relationship between the tissue changes and the predominant micro-organisms. Of the 27 ears examined, 11 were infected with *Proteus* or *Pseudomonas* and the remainder with staphylococci, streptococci, diphtheroids and *Pityrosporum*. The number of ears so infected and their association with the commoner tissue changes in otitis are shown in Table 1. Although the number of ears

TABLE I

TISSUE CHANGES IN INFECTED EXTERNAL EARS

Tissue reaction	*Number of infected ears showing tissue changes*	
	Pseudomonas or Proteus (11 ears)	*Other bacterial species and yeasts (16 ears)*
Hyperkeratinization and hypertrophy of the epidermis	11	15
Marked decrease in the activity of the sebaceous glands	6	8
Marked distension of the excretory ducts of the ceruminous glands ..	10	8
Marked fibroplasia	9	7
Increased number of tissue mast cells in the corium	9	5

examined was small, the results in Table 1 suggest that tissue changes such as distension of the tubular parts of the ceruminous glands, marked fibroplasia and a greatly increased number of tissue mast cells are commoner in ears infected with *Proteus* or *Pseudomonas* than with other micro-organisms.

DISCUSSION

Histologically, the canine external ear closely resembled that of man and other animals. Among its few distinguishing features were the large, actively secreting sebaceous glands in the superficial dermal layers which were especially numerous in the more distal parts of the external ear canal; whereas the modified ceruminous

glands were extremely small and were, apparently, confined to the deeper layers of the hypodermis. The ceruminous glands were thought to be responsible for maintaining the fluid consistency of the wax, produced by the sebaceous glands, thus protecting the intact horny layer of the lining epithelium from external irritants and infection. By confirming the presence of both types of gland in the external ears of healthy dogs these observations refute the suggestion of Miller and Witter (1942) and Witter (1949), that ceruminous glands occur only in diseased ears.

The external ear canals of dogs suffering from various forms of otitis were readily distinguished histologically from those of healthy dogs. Hypertrophy and hyperkeratinisation of the surface epithelium and hair follicles were among the earliest changes observed but, as the disease progressed, changes were also seen in the state of activity and distribution of the glandular structures. In most chronically affected ears the sebaceous glands were smaller and less active than in healthy ears and tended to be displaced from the superficial dermal layers by the excretory ducts of the modified ceruminous glands which were grossly distended with an accumulation of eosinophilic, homogeneous colloidal material. The altered appearance of the glandular tissues was usually associated with changes in the nature of the secretions within the external acoustic meatus. In long-standing cases of otitis, in which distension of the ducts of the ceruminous glands was pronounced, the secretions were copious, moist, and pale-yellow in colour, or of a more purulent nature if infected with *Pseudomonas, Proteus* or other pyogenic bacteria. In cases of acute otitis, however, most of which were infected only with staphylococci or *Pityrosporum*, there was usually little alteration in the appearance of the exudates or in the histology of the ear, other than thickening of the epithelium.

Of the other tissue changes observed, ulceration of the lining membrane, tumour formation and marked fibroplasia, with constriction of the lumen of the auditory canal, were mostly confined to chronically affected ears.

CONCLUSIONS

Histologically, the external auditory meatus of healthy dogs is characterised by the presence of numerous, large, actively secreting sebaceous glands in the superficial dermal layers and occasional, small, tubular apocrine glands in the deeper parts of the corium.

In affected ears, the ducts of the apocrine glands are frequently distended with eosinophilic, colloidal material and tend to displace the sebaceous follicles in the superficial layers of the hypodermis.

The glandular tissue reactions may determine the nature of the secretions within the external ear canal and are mostly related to the duration of the clinical condition or to infection with *Proteus, Pseudomonas* or other pyogenic Gram-negative bacilli.

ACKNOWLEDGMENTS

Thanks are due to Mr. K. W. Head for helpful advice and to Mr. R. C. W. S. Hood for the photography.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Section of skin near the opening of the healthy external auditory meatus, showing the thin epidermis, occasional hair follicles, adipose tissue in the hypodermis, large actively secreting sebaceous follicles in the superficial dermis and occasional small tubular apocrine glands below. × 36.

Fig. 2. Section of the healthy external auditory meatus between the tympanum and the 'angle' of the external ear canal, showing occasional hair follicles and their associated sebaceous glands, the compact connective tissue in the corium and the dense fibrous attachment to the annular cartilage. × 32.

Fig. 3. Chronic, non-suppurative otitis, infected with staphylococci and *Pityrosporum*. There is hyperkeratinization and hypertrophy of the lining membrane and hair follicles, while the sebaceous glands appear to be less active than those of healthy ears. × 36.

Fig. 4. Chronic suppurative otitis with perforation of the tympanic membrane, associated with *Proteus mirabilis* infection. The section shows the bloated appearance of the cells in the stratified squamous epithelium, exaggerated 'rete-peg' formation due to proliferation of the cells in the stratum germinativum, and the dermal papillae which are extensively infiltrated with polymorphs. × 29.

Fig. 5. Chronically affected ear with a mixed bacterial flora including staphylococci, streptococci and corynebacteria. Proliferation of the fibrous tissue extends from the deeper dermal layers throughout the corium, forming tumour-like masses which tend to occlude the lumen of the external ear canal. × 6·5.

Fig. 6. Subacute otitis with a mixed bacterial flora. Distended tubular ducts of the apocrine glands appear alongside the actively secreting sebaceous follicles. × 20.

Fig. 7. Subacute otitis infected with *Proteus mirabilis*. The sebaceous glands are less active and the distended apocrine secretory tubules are filled with plaques of colloidal material. × 26.

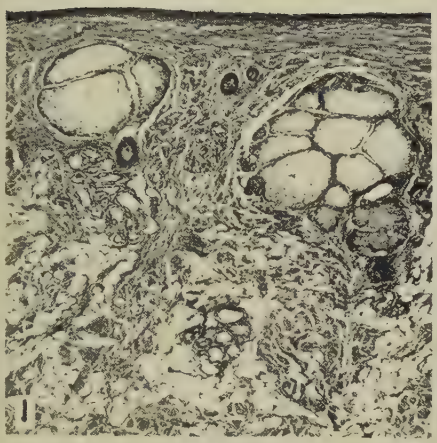
Fig. 8. Chronic otitis of two-years duration with a mixed bacterial infection. Numerous grossly distended secretory tubules of the apocrine-type glands appear to have completely displaced the sebaceous follicles in the superficial dermal layers. × 30.

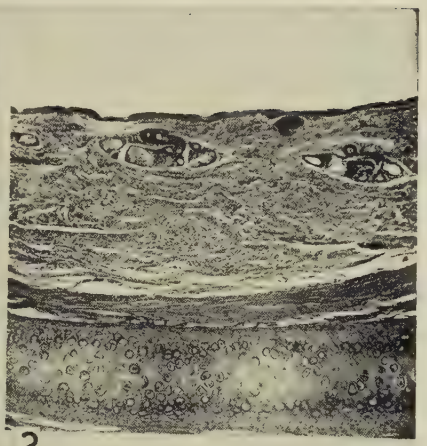
Figs. 9 and 10. Chronically affected ears. The secretory portion of the apocrine gland reaches the surface of the skin alongside the orifice of a hair follicle (Fig. 9. × 29) or a short distance from it (Fig. 10. × 34).

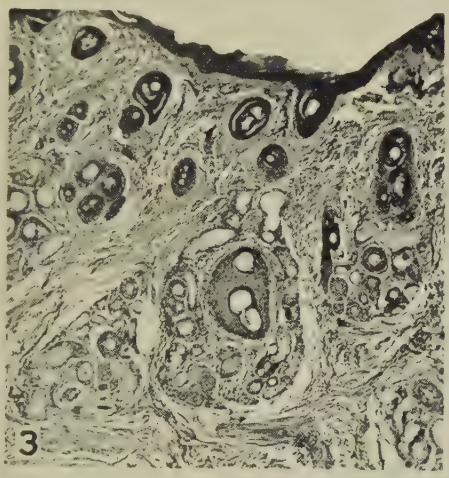
Fig. 11. Adenocarcinoma of the ceruminous glands in a 14 year old Spaniel suffering from external and middle ear disease. The lining membrane gives rise to papillary projections resembling the pattern of an intraductal carcinoma of the mammary gland. × 12.

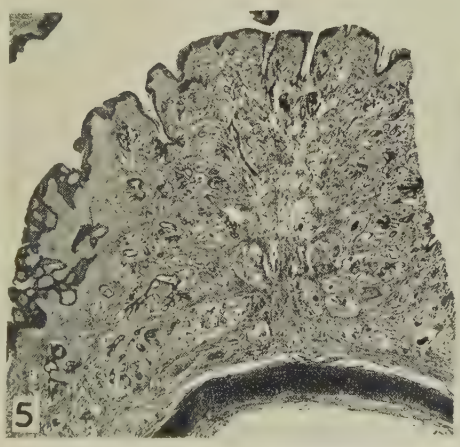
Fig. 12. Tumour of the sebaceous glands in an eight year old Border Terrier, showing the distribution of the hyperplastic sebaceous follicles and proliferation of the connective tissue. × 16.

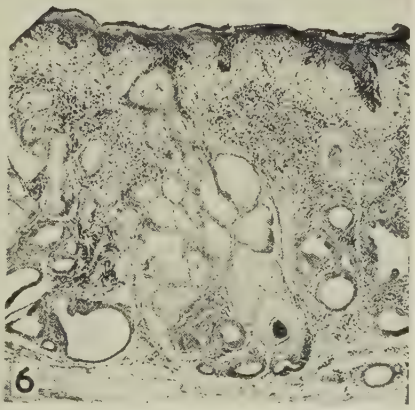
All sections were stained with haematoxylin-eosin.











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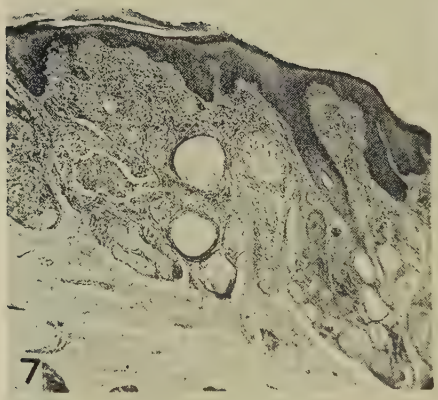
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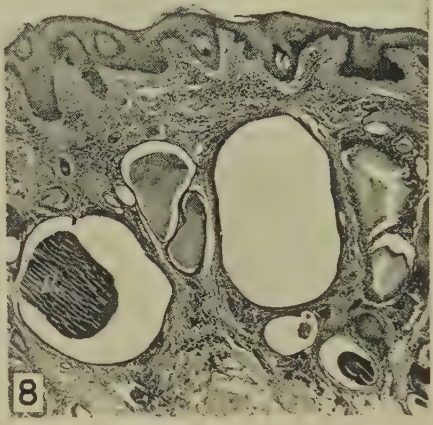
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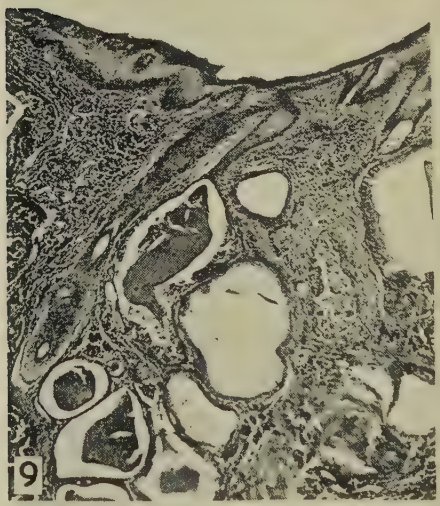
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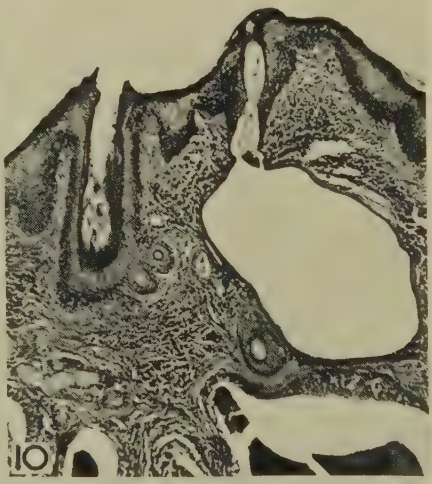
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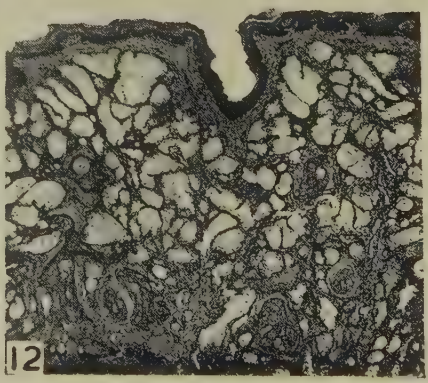
12

SOLID MEDIA FOR THE GROWTH OF *ASTEROCOCCUS MYCOIDES*

By

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INTRODUCTION

One of the factors limiting the study of *Asterococcus mycoides* is the unsuitability of most solid media for promoting rich and regular growth. Despite some reports in the literature of suitable media (Nakamura, Futamura and Watamiki, 1926; Bennett, 1932; Turner, 1953), the personal experience of the author and that of Priestley (Priestley, 1952) in the Sudan have repeatedly shown that growth on the well-known solid media, when obtained, was often irregular and usually poor. This, coupled with the fact that this field, important though it is, has received little attention in recent years, has stimulated the author to conduct the trials embodied in this paper with a view to devising simpler and better methods than those now available for isolating and counting the organism and studying its antigenic properties.

MATERIALS AND METHODS

Basic Media

Four basic media have been prepared and tested for growth. Medium A is a nutrient agar prepared from minced beef heart extract. Medium B is a liver agar prepared from minced bovine liver extract. Medium C is a mixed mince agar prepared from the extract of a mixture of equal weights of minced heart and minced liver. Medium D is a mixed broth agar prepared from a mixture of heart and liver broths either made separately and mixed prior to the addition of agar or mixed soon after extraction.

Medium A (*nutrient agar*). Cleaned beef hearts were minced, weighed and mixed with double amounts (W/V) of distilled water and refrigerated after thorough stirring for 24 hours. The mixture was then boiled for 30 minutes, strained through fine muslin and filtered through paper pulp. Peptone (2 per cent) and sodium chloride (0·5 per cent) were added to the filtrate which was then heated to dissolve the ingredients; the pH was adjusted to 7·6, 2·5 per cent agar added and the mixture steamed for 30 to 40 minutes until the agar melted. The pH was brought up to 7·6. The medium was then steamed for 30 minutes, filtered through paper pulp, bottled in 15 ml. amounts and finally autoclaved at 10 lb. pressure for 30 minutes.

Medium B (*liver agar*). The method adopted was the same as in medium A except that minced liver was substituted for minced beef heart.

Medium C (*mixed mince agar*). This consisted of a mixture of equal weights of minced heart and liver to each of which double the amount of distilled water was added. The mixture was left in the refrigerator for 24 hours. The rest of the procedure was the same as that described for media A and B.

Medium D (mixed broth agar). Heart and liver were minced and extracted separately in double amounts of distilled water in the refrigerator for 24 hours. Each mixture was then boiled for 30 minutes and strained through muslin. To the filtrates 2 per cent peptone and 0·5 per cent sodium chloride were added. The mixtures were boiled until the ingredients dissolved and after cooling, the pH was adjusted to 7·6. Equal volumes of the broths were now mixed, 2·5 per cent agar added and the mixture steamed for about 30 minutes to dissolve the agar. Thenceforwards the procedure was identical with that described for the preceding media.

The method was subsequently modified. After separate extraction in the refrigerator for 24 hours, boiling and straining, the two filtrates were mixed in equal volumes and filtered through paper pulp. Peptone (2 per cent) and sodium chloride (0·5 per cent) were added and the rest of the procedure carried out as described.

In every case the media used in making any one comparative test were all prepared from the same lots of minced hearts and liver.

Supplements

Sera. Sterile ox, horse or sheep sera in concentrations of 10, 20 or 30 per cent were included in the media.

Desoxyribonucleic acid (D.N.A.).

Glucose, yeast extract and egg yolk. These substances were employed in some trials in the final concentrations given in the respective tables. Stock solutions (5 per cent) of the first three substances were prepared in distilled water and sterilized by Seitz filtration before storing in the refrigerater. Egg yolk was recovered under sterile precautions from infertile eggs of White Leghorn hens.

Penicillin. Solution of crystalline sodium penicillin G in sterile broth was regularly included in all media at a final concentration of 30 I.U/cc.

Test Strains of the Organism

Strain 121/1–2 (Virulent) which was originally a dried muscle tumour exudate was cultured once or twice in 10 per cent horse serum broth and was used in this form in most experiments as 2 to 4 days old cultures.

Strain FK 91–93 (attenuated) was a vaccine strain which had been subjected to 91 to 93 weekly subcultures in serum broth after initial isolation. It was used in some experiments as 2 to 4 days old cultures

Strain 121/7 was partially attenuated by seven subcultures after isolation. It was subsequently dried, kept in the refrigerator for over a year, and was seeded immediately after reconstitution in nutrient broth.

Natural material was used in the form of exudate recovered from an artificial muscular swelling and seeded direct to the plates within one week of storage in the refrigerator.

Techniques

Supplementary ingredients, including serum, were added to the melted media after cooling to about 55°C. The media were then poured into petri dishes and allowed to set at room temperature. Seeding in all cases, except for counts, was effected by spreading one loopful of the test material on the surface of the medium. The plates were then incubated at 37°C. and read daily for periods of up to one week.

For the single experiment on counts, serial tenfold dilutions of the culture were made in 10 per cent horse serum broth. By means of a

fiftieth ml. dropper, drops from dilutions of 10^{-6} to 10^{-10} were placed on sectors of an agar plate. After incubation for seven days the tubes and the plates were compared.

RESULTS

Growth on Media A, B, C and D (in the presence of 10 per cent and 20 per cent horse serum and penicillin)

The results are set out in Table 1. It is clear that 20 per cent serum gave better growth than 10 per cent and that both with 10 per cent and 20 per cent serum, medium D gave the best results. There was no difference between medium C and nutrient agar with 10 per cent serum but 20 per cent serum produced better growth on the former. Liver agar occupied the bottom position. Repetition of the trial with other batches of media from the same meat sources gave consistently similar results indicating that medium D was superior to the other three.

Fig. 1(a) shows the type of growth obtained with the two strains on medium D. Fig. 1(b) is a field of the richer half of the plate as seen under the low power objective (32 mm. lens).

Effect of Three Concentrations of Horse, Ox and Sheep Sera (Medium D)

Table 2 shows the progressive increase in growth with increasing concentration of any of the three sera. While it is evident that ox and horse sera, on the one hand, produced better growth than sheep serum, there was an indication that ox serum was slightly better than horse serum. This indication was strengthened by the additional observation that the size of colonies on the ox serum plates appeared a little larger than those on the horse serum plates.

Effect of Desoxyribonucleic Acid plus 30 per cent Ox Serum (Medium D)

The results (Table 3) show relatively richer growth on the plates containing D.N.A. than on the one without D.N.A. It was also observed that the colonies on Figs. 1, 2 and 3 were appreciably larger (about one and a half times) than those on plate 4. For rough comparison of growth see Fig. 2(a) which contained D.N.A. and Fig. 2(b) without D.N.A. The table also suggests that for the amounts used in the experiment the optimum concentration of D.N.A. appears to fall around 0·06 per cent.

Effect of Various Combinations of D.N.A, Egg Yolk, Yeast Extract and Glucose on Growth on Medium D with 30 per cent Ox Serum.

The results of this experiment using strain 121/1 are shown in Table 4. The general impression was that differences where they existed were not large. It appeared that (1) in the presence of D.N.A. no obvious advantage resulted from the addition of yeast extract, glucose or a combination of both: (2) yolk alone slightly improved the growth but to a less extent than D.N.A. alone: (3) the presence of yeast extract, but not glucose, with yolk, augmented growth to an extent comparable with that produced in the presence of D.N.A.

TABLE 1

GROWTH ON MEDIA A, B, C AND D

In the presence of 10 and 20% horse serum and penicillin

Medium	*Strain*	*10% horse serum growth after (days)*							*20% horse serum: growth after (days)*						
		1	*2*	*3*	*4*	*5*	*6*	*7*	*1*	*2*	*3*	*4*	*5*	*6*	*7*
A. Nutrient agar	FK 91	–	–	–	+	+	+	+	–	–	+	+	+	++	++
	121/1	–	–	–	+	+	+	+	–	+	+	+	+	++	++
B. Liver agar	FK 91	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	121/1	–	–	–	–	+	+	+	–	+	+	+	+	+	+
C. Mixed mince agar	FK 91	–	–	+	+	+	+	+	–	–	+	++	++	++	++
	121/1	–	–	+	+	+	+	+	–	+	+	++	++	++	++
D. Mixed broths agar	FK 91	–	–	+	+	+	++	++	–	+	++	++	++	++	++
	121/1	–	–	+	+	++	++	++	–	+	++	++	++	+++	+++

Key To Tables 1 to 5

– no growth after seven days.
+ scanty growth with tiny scattered colonies hardly visible to the naked eye.
++ numerous crowded colonies where inoculum is thick but little or no growth where inoculum thins out along the track of the loop.
+++ confluent growth where inoculum is thick and rich growth along the whole track of the loop.

TABLE 2

EFFECT OF THREE CONCENTRATIONS OF HORSE, OX AND SHEEP SERA (MEDIUM D)

Serum conc.	*Donor*	*Strain*	*Growth in days*						
			1	*2*	*3*	*4*	*5*	*6*	*7*
10%	Horse	FK/91	—	—	—	—	—	—	—
		121/1	—	—	—	—	+	+	+
	Ox	FK/91	—	—	—	—	—	+	+
		121/1	—	—	—	+	+	+	+
	Sheep	FK/91	—	—	—	+	+	+	+
		121/1	—	—	—	—	+	+	+
20%	Horse	FK/91	—	+	+	+	+	++	++
		121/1	—	+	+	++	++	++	++
	Ox	FK/91	—	+	++	++	++	++	++
		121/1	—	+	++	++	++	++	+++
	Sheep	FK/91	—	—	—	+	+	+	+
		121/1	—	—	+	+	++	++	++
30%	Horse	FK/91	—	+	++	++	+++	+++	+++
		121/1	—	+	++	+++	+++	+++	+++
	Ox	FK/91	—	+	++	+++	+++	+++	+++
		121/1	—	++	++	+++	+++	+++	+++
	Sheep	FK/91	—	—	+	++	++	++	++
		121/1	—	+	+	++	++	++	++

TABLE 3

EFFECT OF DESOXYRIBONUCLEIC ACID (MEDIUM D)

Plate No.	*% D.N.A.*	*Growth in days*			
		1	*2*	*3*	*4*
1.	0·25	—	+	+++	+++
2.	0·125	—	++	+++	+++
3.	0·0625	—	++	+++	+++
4.	—	—	+	++	++

Results with Modified Medium D

The simplified formula referred to earlier under 'Medium D' was compared for growth with a parallel batch prepared in the original way. A plate from each lot containing 30 per cent horse serum was sown with strain 121/2 and examined for growth. The

TABLE 4

EFFECT OF VARIOUS COMBINATIONS OF D.N.A. EGG YOLK, YEAST EXTRACT AND GLUCOSE WITH 30% OX SERUM (MEDIUM D)

Plate no.	*D.N.A.* %	*Egg yolk* %	*Yeast ext.* %	*Glucose* %	*Growth days*				
					1	*2*	*3*	*4*	*II*
1	0·06	—	—	—	—	++	+++	+++	+++
2	0·06	—	0·1	—	—	++	+++	+++	+++
3	0·06	—	—	0·1	—	++	+++	+++	+++
4	0·06	—	0·1	0·1	—	++	+++	+++	+++
5	—	2·5	—	—	—	+	+++	+++	+++
6	—	2·5	0·1	—	—	++	+++	+++	+++
7	—	2·5	—	0·1	—	+	++	++	+++
8	—	2·5	0·1	0·1	—	+	+++	+++	+++
9	0·062	2·5	0·1	0·1	—	++	+++	+++	+++
10	—	—	—	—	—	+	++	+++	+++

TABLE 5

GROWTH ON UNMODIFIED AND MODIFIED MEDIUM D WITH 30% HORSE SERUM

	Growth in days				
	1	*2*	*3*	*4*	*5*
Unmodified medium D	−	+	++	+++	+++
Modified medium D	±*	++	+++	+++	+++

*Ground glass appearance on the thick part of the inoculum.

results (Table 5) show that simplification of the process has resulted in an advantage. How far it will be possible to modify the method still further will be the subject of a subsequent investigation.

It should be mentioned in this connection that it was hoped that medium C, which chronologically succeeded medium D would provide the answer, but for some reason unknown to the writer, the results obtained with it were in some instances disappointing when compared with those consistantly produced with medium D. One such instance is illustrated in the Fig. 3(a) and Fig. 3(b) which show 7 days growth on a batch of media C and D respectively prepared from the same meat sources and inoculated at the same time from one tube of culture. This of course does not exclude the possibility of better results attending improvement of the method of preparation of medium C.

Use of Medium D for Isolation of the Organism

The value of the above medium for the isolation of the organism is illustrated by Figs. 4 and 5. The former shows growth of exudate from a muscular swelling. The latter is the growth of strain 121/7 seeded immediately after reconstitution with nutrient broth. The advantage of this method is that it does away with the relatively cumbersome practice of having to pass the inoculum from pathological material or dried culture through a series of dilutions of liquid medium in order to ensure exclusion of contaminants.

Use of Medium D for Counts

The possibility of using medium D for counts was investigated in one experiment carried out by the method described under 'Techniques'. Fig. 6 shows growth on sectors seeded with drops from dilutions of 10^{-6} to 10^{-10}. It so happened, however, that the serum broth dilution series gave positive growth up to 10^{-10}, the last dilution in the series. It was not possible therefore to compare end points. Nevertheless, the evidence from the figures was an indication of the possibility of using this medium for counts.

DISCUSSION

The use of 30 per cent serum was mentioned earlier by Nakamura *et al.* (1926). In 1947 Edward used 20 per cent serum for the growth of PPLO organisms. More recently, Australian workers (1960) have reported the use of BVF-OS digest medium solidified with 2 per cent agar and incorporating 30 per cent ox serum for counts of *Asterococcus mycoides*.

The present work offers a preliminary survey of some comparatively simple media with the object of selecting the most promising and subjecting it to a more concentrated study. In the experience of the author medium D proved to be very satisfactory. Nutrient agar and liver agar respectively were useful, particularly when enriched by the addition of 20 per cent serum or more. It is interesting to note the difference between these two media and medium D which, in effect, constitutes a mixture of the two. The difference was more noticeable with 10 per cent serum. It was however, observed that with both 10 per cent and 20 per cent horse serum, Medium D in one experiment was not entirely satisfactory. This may have been due to the fact that the batch of medium used was sown after nearly four weeks of storage, in screw capped bottles, during September when the average temperature is over 37°C. The author does recall more than one instance of deterioration following long storage. However, since the object of the experiment was intended to be a comparison of sera and not media, the age of the batch was not considered critical in the experiment in question.

Edward and Fitzgerald (1951, 1952) refer to desoxyribonucleic acid and cholesterol as being essential substances for pleuropneumonia like organisms. Edward (1947) also used yeast extract to promote growth of these organisms. It appears from the results obtained that though medium D without these substances was capable of sustaining good growth, the inclusion of D.N.A. or of egg yolk and yeast extract combined enhanced the growth and may thus be useful when using the medium for counts. No similar growth-promoting effect was noted with glucose. Whether this was due to the reducing effect of the sugar or to the acidity resulting from its fermentation by the organism or both is a point which may be usefully investigated. It may be worthwhile finding out whether the addition of a buffer is an advantage.

By using the medium, the author and Dr. Abdel Aziz El Tayeb of this Faculty successfully isolated a PPLO from the milk of goats suffering from mastitis and Dr. C. P. Pillai (personal communication) isolated a PPLO from the lungs and chest exudate of goats suffering from pleuropneumonia. These two findings suggest the possibility of using the medium for the isolation of other PPLO organisms.

Finally, studies on antigenic structure have so far been conducted with suspensions subjected to varying degrees of subculture in fluid media. What we consider to be an attenuated culture may in fact be

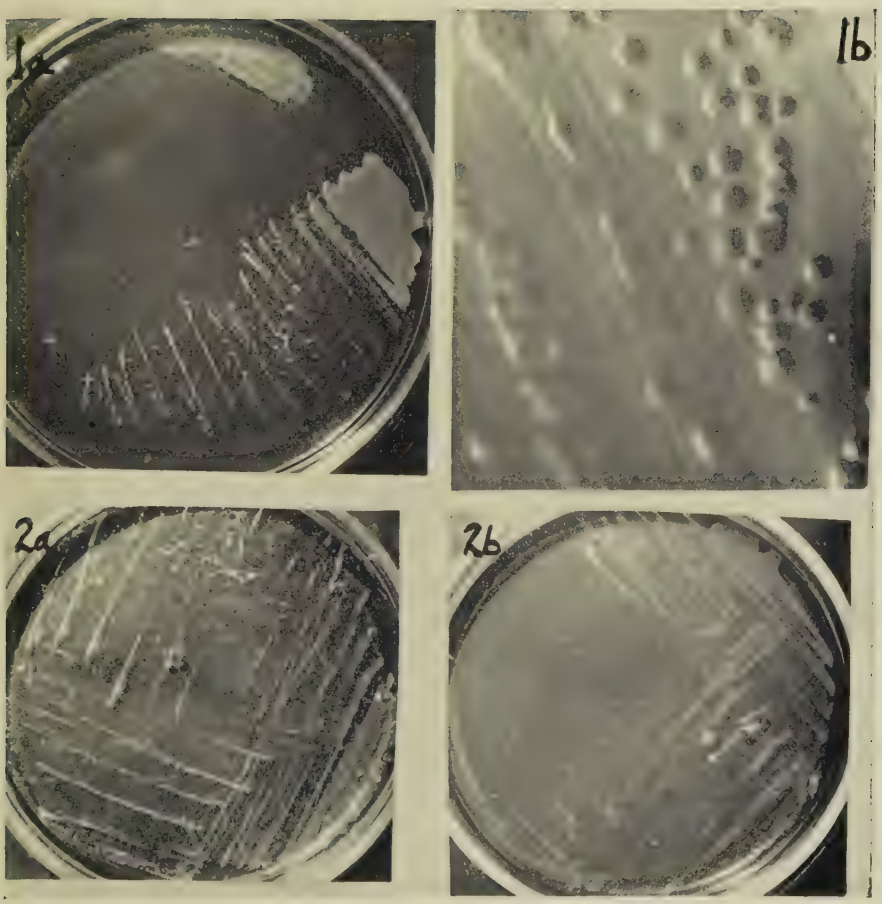

Fig. 1a Growth of two strains on medium D.

Fig. 1b PPLO colonies: low power (32 mm. lens).

Fig. 2a 7 day culture medium D + 0·125 D.N.A.

Fig. 2b 7 day culture medium D without D.N.A.

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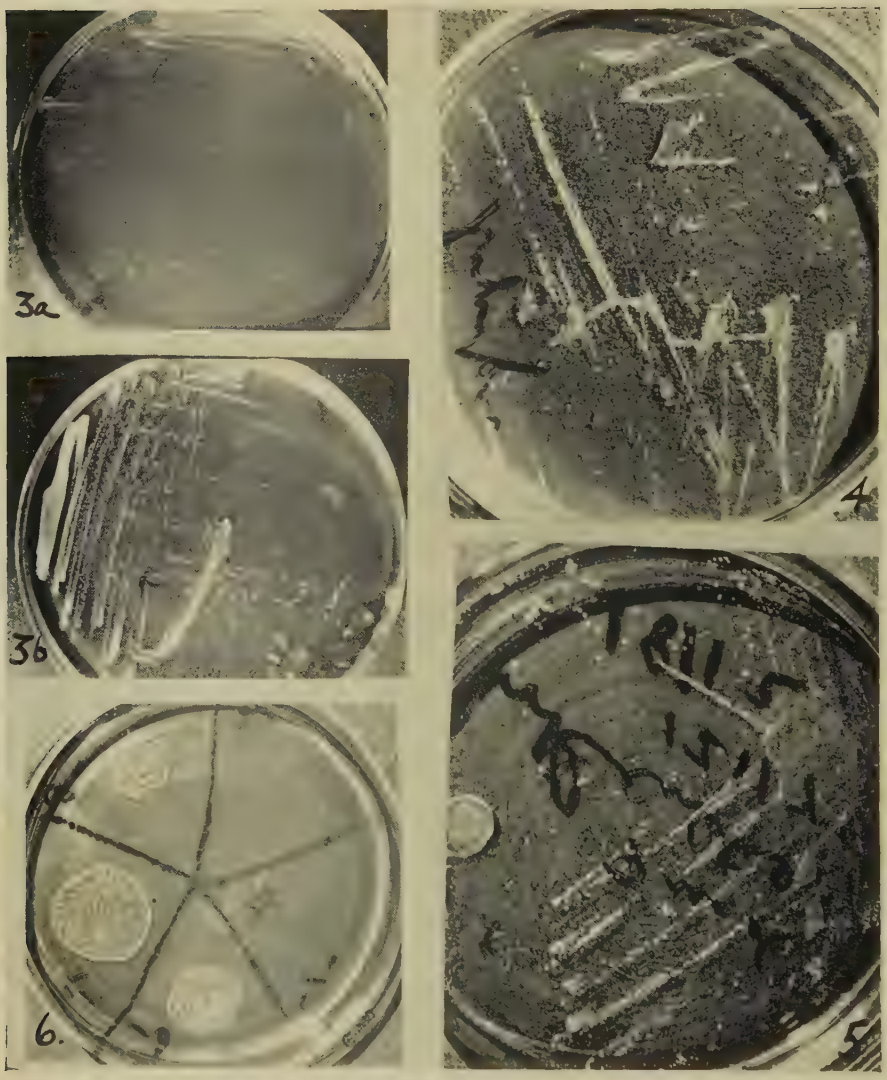

Fig. 3a 7 day culture strain 121/2 on medium C.

Fig. 3b 7 day culture strain 121/2 on medium D.

Fig. 4. 7 day culture from exudate.

Fig. 5. 7 day culture from dryculture 121/7.

Fig. 6. Value of medium with D.N.A. for counts.

a mixture containing a low proportion of virulent cells. They might not be numerous enough to cause lesions but their presence might mask a serological difference between two phases. To avoid this complication, it is desirable to work with suspensions of cultures isolated from single colonies and for this purpose, as well as for a more thorough study of colonial variation in relation to immunity, a reliable solid medium is essential.

CONCLUSIONS

Solid media prepared from extracts of ox heart and ox liver and supplemented with varying concentration of ox, horse and sheep sera have been described and tested for the growth of *Asterococcus mycoides* in the presence and absence of desoxynucleic acid, egg yolk, yeast extract and glucose.

The results of several experiments show that a medium (D) in which heart and liver were extracted separately and subsequently mixed to form the base, gave the best results. Other media were less satisfactory but were improved by the addition of higher concentrations of sera.

Of the three sera used, ox serum appeared to give slightly better results than horse serum and sheep serum was inferior. In all cases the best of the three concentrations tested was 30 per cent followed by 20 per cent and then 10 per cent.

D.N.A. and, to a less extent, egg yolk accelerated the growth. A combination of egg yolk and yeast extract gave results comparable with those produced by D.N.A. alone. Glucose did not appear to improve the growth.

The uses of a solid medium for the primary isolation of PPLO and for counts of small numbers are discussed.

ACKNOWLEDGMENT

I am greatly indebted to Dr. M. N. Hamza without whose help the completion of this work would not have been possible. I am grateful to Professor T. J. Bosworth for revising this paper. I also thank Ustaz Abdel Aziz El Tayeb and Ali Eff. Mohammed Saad for their assistance.

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ANTIBODY CONTENT OF COLOSTRUM AND PIGLET SERUM FOLLOWING VACCINATION OF THE SOW

By

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INTRODUCTION

The persistent recurrence of the condition of gut oedema (oedema disease) in a herd of swine used for nutritional experiments made it necessary to try to develop measures for protecting young pigs against the disease.

A previous report by Lemcke, Hirsch and Bellis (1957) noted the association of haemolytic *E. coli* with the disease in this herd; this association is in agreement with the observations of other workers (Schofield and Davis, 1955; Gregory, 1955; Timoney, 1956; Sojka, Erskine and Lloyd, 1957). The young pigs seemed most susceptible to the disease 7 to 14 days after weaning (Lloyd, 1957; Schofield and Nielson, 1955).

It is not clear at present whether vaccination can provide a measure of protection against *E. coli:* so far it has met with varying success. Schofield and Davis (1955) failed to protect pigs with an *E. coli* vaccine prepared from strains isolated from active cases of oedema disease. In contrast, Gordon and Luke (1958) established the association of a non-haemolytic *E. coli* with gastro-enteritis in swine and by vaccinating sows with a heat-killed preparation reduced the mortality in litters obtained at subsequent farrowing. Other experiments are described by Timoney (1957). He inoculated pigs with toxic bowel extract obtained from cases of oedema disease and reproduced the disease. Pigs could be protected from these toxic effects with immune sera from other pigs repeatedly inoculated with alum-precipitated material from haemolytic *E. coli* cultures or from bowel supernatants of pigs with oedema disease.

In the work reported here the sows were vaccinated before farrowing to try to increase the amount of protective *E. coli* antibody available to the young pigs by passive transfer through the colostrum. The antibody content of samples of colostrum from the sows and blood from the young pigs was determined by agglutination tests, and the mortality rate of young pigs in the different test groups were compared.

EXPERIMENTAL PROCEDURE

Strains of E. coli

The laboratory vaccine was prepared from two distinct serological types of *E. coli* most frequently isolated from the herd. One of these has been identified by W. Sojka (Ministry of Agriculture, Veterinary Laboratory, Weybridge) as being the same as Weybridge serotype E4 (Sojka *et al.*, 1957), the type which was most frequently associated with oedema disease. The antigenic pattern of our strain was determined at the International Salmonella and Escherichia Centre, Copenhagen, and found to be O 139-H1. In this paper, the Weybridge number E4 is used for this

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strain. The other strain is called RS1 in this paper; it could not be definitely identified with other published strains. Boiled broth cultures of RS1 reacted with Weybridge OKG 43 serum and the International Coli Centre placed it into group O4.

Vaccines

Preparation. Two vaccines were used, a commercial polyvalent *E. coli* vaccine (C), and a laboratory-prepared vaccine (L). Vaccine L was prepared from 24 hour cultures of strains E4 and RS1 grown in Difco heart-infusion broth. Cells were harvested from 1 litre of broth by centrifugation, washed twice and resuspended in 50 ml. normal saline; 50 ml. amounts of each of the two cultures were mixed and commercial formalin was added to a final concentration of 0·6 per cent.

Administration. Sows due to farrow were randomly divided into three groups; two received different vaccines and the third was the unvaccinated control group. Sows were injected with 10 ml. of vaccine C or L intramuscularly 4 weeks before the estimated date of farrowing. Sows allocated to the control group were not injected.

Pigs

A total of 62 sows which gave birth to 642 pigs were examined. The sows were either Essex or Essex/Large White crosses. They were kept in a confined farrowing house and most of the piglets were "early weaned" at about 12 days of age when they were transferred to another special house. The others were weaned at 8 weeks. The pigs intended for slaughter were fed a proprietary food containing a tetracycline antibiotic at 40 g/ton.

The number of deaths during the first 28 days of life was recorded for each of the 62 litters. This period was chosen because, for early-weaned pigs at least, the most critical period is the week after weaning.

Samples for in Vitro Tests

Colostrum. Samples of colostrum were obtained just before or just after farrowing. After removing fat by centrifugation, 1 per cent rennet was added to the aqueous fraction which was then incubated at 37°C. for 30 minutes. After centrifugation twice more, a clear or slightly opalescent preparation was obtained. It was necessary to remove as much fat as possible before adding rennet in order to obtain a clear preparation.

Aliquots of a colostrum sample were absorbed separately with strains E4 and RS1 to see whether the agglutination observed in colostrum was due to specific or non-specific factors. Absorption was carried out in two stages. First, 1 ml. of colostrum prepared as described above, was incubated for 1 hour at 50°C. with 4·5 ml. of a thick cell suspension of the appropriate strain of *E. coli*. The cell suspension was prepared in physiological saline from the 18 hour growth on 0·1 per cent glucose agar plates. After centrifugation a second absorption was made with a further 4·5 ml. of a similar suspension. The colostrum (diluted 1 in 10) was finally centrifuged twice to free it as completely as possible from the absorbing cells.

Serum. Pigs were bled from an ear vein, about 5 ml. amounts of blood being taken at any one time. Samples were refrigerated overnight and the serum separated by centrifugation. Phenol (0·3 per cent) was added as preservative. Blood samples were taken from selected pigs from thirteen litters (4 litters in the "C" vaccine group, 5 in the "L" vaccine group and

4 in the unvaccinated control group). The first samples were taken before weaning at 9 to 13 days of age, and further samples were taken 7 to 10 days after weaning. Pigs weaned at 8 weeks were sampled when 21 to 22 days old and again 3 to 4 weeks after weaning.

Agglutination Tests

Serum and colostrum samples were tested in duplicate. They were serially diluted (2-fold dilutions) and titrated against various antigen preparations. Two types of antigen were prepared for agglutination tests with absorbed and unabsorbed colostrum and with the sera.

Agglutination. Heart-infusion broth cultures incubated overnight were heated in boiling water for 1 hour. The heated cultures were diluted with normal saline to approximately 750×10^6 cells/ml. Equal volumes of suspension were mixed with serum or colostrum in conical Dreyer tubes. Titres were read after incubating overnight at 48 to 50° C.

Living cell agglutinations. Growth from 0·1 per cent glucose agar slopes was washed off and diluted in normal saline to approximately $4{,}000 \times 10^6$ bacteria per ml. One drop (0·04 ml.) of such a suspension was added from a standard dropping pipette to each 0·5 ml. aliquot of the appropriate serum dilutions in $3'' \times \frac{3}{8}''$ test tubes. The tubes were incubated for 2 hours at 37° C. and allowed to stand overnight at room temperature before reading. Tubes in which the cells collected as a granular deposit at the bottom of the tube and did not disperse on shaking, were read as positive. Saline controls were always included and in every series of agglutinations the antigens were titrated against a strain E4 rabbit antiserum as a further control. It was believed that agglutination of living cells would result from the interaction of K antigen with the corresponding antibody, but the presence of H antigen in both strains E4 and RS1 undoubtedly accounted for some of the interaction observed.

For the purpose of statistical analysis, the greatest dilution giving agglutination was transferred to a logarithmic scale, taking 1/25 as 1, 1/50 as 2, and so on up to 1/51,200 which was taken as 12. The transformed values were statistically analysed.

RESULTS

Serological Tests

Agglutination titres with absorbed colostrum. The results after absorption with *E. coli* strains E4 and RS1 are given in Table 1. Absorption

TABLE 1

ABSORPTION OF COLOSTRUM WITH E. Coli STRAINS E4 AND RS1

Titres (reciprocal of the highest dilution giving agglutination)

Antigen	*Unabsorbed*	*Ansorbed with strain E4*	*Absorbed with strain RS1*
Living E4	5120	40	2560
Living RS1	640	640	40
O E4	80	20	80
O RS1	160	160	20

with one or other of the strains reduced the titres of agglutinations against both heated and living cells of the homologous strain but not of the other. Although absorption by living cells of strain E4 was incomplete the titre was considerably reduced whereas that of strain RS1 was not. It appears that the observed agglutination in diluted colostrum was due to specific agglutinins.

Agglutinin titres with non-absorbed colostrum. Agglutin titres against suspensions of *E. coli*, strain E4 are shown in Fig. 1 and against strain RS1 in Fig. 2. It is evident that the titres were higher in the L vaccine group than in the C vaccine or control groups. Statistical analysis of the means of these values is given in Table 2. Agglutinins against the heated cells of strain E4 were present in such small amounts in the colostrum of 29 out of 62 sows that the results could not be analysed and are not included in Table 2, although there is some indication that vaccine L gave slightly higher concentrations of agglutinins against this antigen also.

TABLE 2

COLOSTRUM SAMPLES

Mean values of agglutinin titres (transformed to a log scale) against three antigens, and average significant difference.

Test antigen	*Living E4*	*Living RS1*	*O RS1*
Vaccine L	12·04	9·36	3·71
Vaccine C	9·17	7·2	3·15
Control	7·95	6·48	2·60
Difference required for significance (P = 0.05)	1·20	0·95	0·52

Table 2 shows that vaccine L gave a significantly higher value against each of the three antigens than either vaccine C or the controls. Vaccine C gave a significantly higher value than the controls for agglutinins against living cells of strains E4 and the O antigen of strain RS1. The difference between vaccine C and the control was a little short of significance when tested against living cells of strain RS1.

A higher concentration of antibody might be expected in the blood and the colostrum of older sows because they would have been exposed for a longer period to the endemic *E. coli* strains, but this could not be proved. The statistical analyses were made on the assumption that there was no such effect.

Agglutinin titres in piglet serum. The differences needed for significance were high because only 5 litters were tested in each group (Table 3). With the "living E4" antigen, the L vaccine group was significantly better than either the control or the C vaccine group. With "living RS1" antigen the L group was significantly better then

Fig 1 AGGLUTININ TITRE OF COLOSTRUM AGAINST E. COLI STRAIN E4

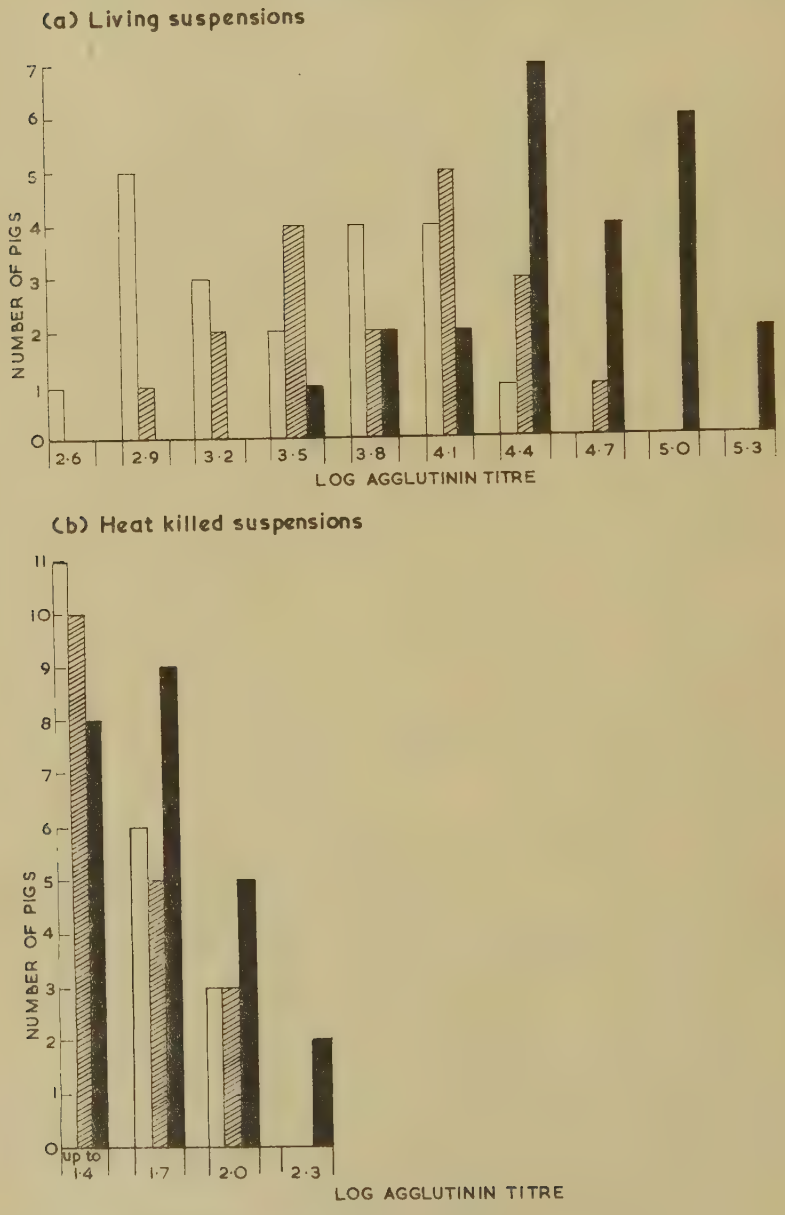
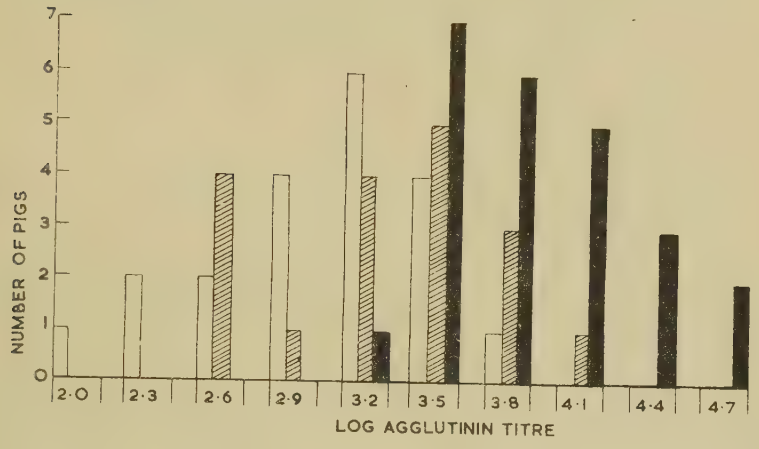
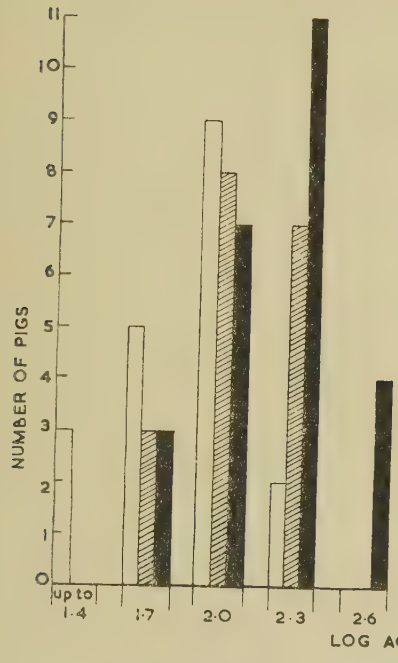


Fig 2 AGGLUTININ TITRE OF COLOSTRUM AGAINST E.COLI STRAIN RS1

(a) Living suspensions



(b) Heat-killed suspensions



= No vaccine = Vaccine C = Vaccine L

TABLE 3

MEAN VALUE OF AGGLUTININ TITRES

Transformed to a log scale of serum of piglets against two antigens.

Test antigen	*Living E4*	*Living RS1*
Vaccine L	10·15	7·82
Vaccine C	6·08	4·73
Control	7·10	5·38
Difference required for significance (P = 0·05)	2·59	2·70

the C group but neither vaccinated group was significantly better than the control.

The action of the agglutinins against the heated cells (O antigen) of both strains was so weak that many of the titres could only be expressed as "less than 50" or "less than 10" and no analysis could be made. However, several samples belonging to the L group gave definite titres, but this reaction was given by only one control pig and none of the C group pigs. Therefore the L vaccine may raise the level of O agglutinins slightly.

There was a suggestion that the titres fell after weaning in the L and the control groups, although there were insufficient samples for statistical analysis. In the L vaccine group this decrease in titre was seen over three successive samplings. The last sample, taken to 3 to 4 weeks after weaning, showed particularly low titres. Although only a few samples were taken from late-weaned pigs a similar trend was indicated.

Mortality Rates

The mortality and its distribution in the different treatments is shown in Table 4. Out of a total of 642 pigs, 550 were early weaned

TABLE 4

MORTALITY AMONG PIGLETS FROM 62 SOWS ON THREE TREATMENTS

Vaccine	*Weaned*	*No. of litters*	*No. of pigs in the litters*	*No. of dead pigs*
L	early	19	195	41
C	,,	16	158	52
Control	,,	18	197	44
L	late	5	51	0
C	,,	2	20	2
Control	,,	2	21	0

(12 days) and 137 died (25 per cent). The remaining 92 pigs were late weaned (8 weeks) and 2 died (2 per cent). For the early-weaned pigs there was very little difference between the mortalities of the L vac-

cine and the control groups. The mortality in the C vaccine group was higher, but statistical analysis showed that this was not more than could have occurred by chance variations between the groups. Although only a relatively small number of pigs was late weaned (8 weeks) the mortality in all three groups was much lower than that of the early-weaned pigs.

DISCUSSION

The colostrum titre of agglutinins against living suspensions of *E. coli* was high, even in the colostrum samples from untreated sows. This was at first suspected to be due to non-specific reactions which were independent of the vaccination. The reaction was, however, shown to be specific because colostrum agglutinins against strains of E4 and RS1 could be separately absorbed.

The presence of such agglutinins might be expected if these strains of *E. coli* were endemic in the premises housing the pig herd. From the date of starting the bacteriological work on the herd, strains E4 and RS1 were known to have been present for at least a year previously. The sows would have been exposed to the strains for at least some period of their lives, as they had all been bred on the premises. Nothing definite was established about the effect of the age of the sows on agglutinin levels although there was a trend for the older sows to have a higher titre.

Irrespective of the vaccination treatment, colostrum samples always gave higher agglutinin titres against living cells of strain E4 than against the living cells of strain RS1; the reverse was true for the heated O antigen. The serum titres were also higher against "living" E4 than "living" RS1, but there was little difference between the corresponding O titres, which were both very low.

Not all agglutinin titres were affected to the same extent by vaccination. Vaccine L increased agglutinin titres of both serum and colostrum against the corresponding O antigens. Vaccine C also increased the agglutinin titres of colostrum against the living cells of strain E4 and the O antigen of strain RS1, but the effect was apparently not passed on to the offspring since their levels of serum agglutinins did not differ significantly from the controls. From this experience with vaccine L and from the low O titres of serum samples irrespective of group, it seems that a very high level of agglutinins is required in the colostrum of the sow if the effect is to be appreciable in the blood of the young pigs.

Vaccine L effectively increased the agglutinins titre of colostrum against three of the four antigen preparations and this increase was reflected in the increased agglutinin titres against two of these antigens in the serum of the offspring. Vaccine C was less effective, but this was to be expected since in a polyvalent vaccine the amount of antigen which would specifically stimulate the production of agglutinins reacting with strains E4 and RS1 was probably small.

The serum agglutinin titres observed in the first examination at 9 to 13 days showed a tendency to fall at subsequent examinations even when the pigs were still sucking the sow. Thus, antibodies passively transferred in the colostrum do not seem to persist for very long. This correlates with the observations of McGirr (1947) that absorption of antibody only occurs in the calf in the first 24 to 48 hours of life; Earle (1935) also showed that an initial rise in the serum globulin of pigs during the first 24 hours of life is followed by a decrease over the next 12 days. More recently Speer, Brown, Quin and Carron (1959) observed that antibody absorption in the baby pig is negligible after the first 24 hours of life. The effects of vaccinating the mother, as judged by agglutinin levels, seem therefore to be available to the offspring for only a very limited period.

Considering the high agglutinin titres in the colostrum of the vaccinated sows and in the blood of their offspring in the early stages of life, the mortality rates were disappointingly high. It seems that either the young pigs died from some cause other than infections with *E. coli*, or that the antibodies detected in the colostrum and serum samples were ineffective. The latter explanation is more acceptable because signs of oedema or gastro-enteritis were observed frequently in the herd at this time and undoubtedly these diseases played a major part in the high incidence of deaths recorded. Moreover there is evidence that another serological type of haemolytic *E. coli* made its appearance on the premises at about the time of the vaccination trial. It was first isolated about a month after the beginning of the vaccination programme and since then it has been found in almost all the pigs examined. It has been identified with the Weybridge type E57 (International Centre typing O 138, K 81) which is reported as being third in frequency of isolation both from cases of swine gastro-enteritis and from cases of oedema disease (Sojka *et al*, 1957). Since its first appearance it has been isolated from our herd more frequently than the commonest gut oedema serotype (strain E4) and it seems to have replaced the latter as the dominant haemolytic *E. coli*. Vaccine L could not provide the specific stimulus for producing antibodies against this strain and would not be effective if the new serotype caused deaths during the experiment.

Even if the serotype represented in the vaccine was still causing disease during the vaccination trial, the inefficiency of the vaccines could be due to a failure to stimulate the production of protective antibodies. Although strain RS1 possesses a K antigen, F. Orskov (International Coli Centre, Copenhagen) has since reported that strain E4 has no K antigen. It could therefore not elicit the production of K antibodies. It has been established by Briggs (1951) that the antibody in cow colostrum which is able to protect calves from "white scours" caused by *E. coli* is the one produced against the K antigen of pathogenic strains. If this is also true for *E. coli* infections in swine, the vaccine would not help to overcome infections, even with *E. coli* strain E4. The agglutination to high titre observed

between living cells of strain E4 and the pig colostrum or serum could have been due to the flagellar (H) antigen. Titres obtained in O and H agglutination reactions are generally higher than those obtained in K agglutination reactions. A better vaccine might have been prepared if a strain of the same serotype as strain E4 but possessing the K antigen had been chosen.

One other point warrants consideration. It seems that young pigs need the protection of the sows' milk over quite a long period, as shown by the lower mortality rate in the pigs suckled for 8 weeks. When this work was being done the pigs were weaned at about 12 days and with this short weaning time the pigs appeared to need protection additional to that given by the brief suckling period. Although 40 g. aureomycin/ton feed was fed, the mortality rate was 25 per cent, which suggests that substances other than antibiotics would have to be used. The coliform flora were evidently antibiotic-resistant (Timoney, 1957).

In order to protect the pigs, a very high antibody content is needed in the colostrum because this is available to the pigs for only a short time. Further, the concentration in the piglet's blood declines rapidly; the high concentrations of antibody shown in Fig. 1 were obtained with pigs sampled at weaning time, but they were not obtained when they were sampled 10 days after weaning.

It is also clear that vaccines have to be effective against the specific organism which is dominant at the time of weaning. There is no certainty that between the time of vaccination and weaning (about 6 weeks) a new pathogenic serotype may not become dominant, as occurred in the present study. The piglet antibodies are then ineffective. The total number of *E. coli* serotypes which are associated with gut oedema, enteritis and other scouring conditions is unknown, but is likely to be large. A polyvalent vaccine aimed at protecting against all these types is likely to fail because such vaccines do not contain sufficient antigen to stimulate antibody production against the dominant *E. coli* serotype; this occurred with the commercial vaccine 'C'.

CONCLUSIONS

Sixty-two sows were divided into three groups; one group was vaccinated with a formalized mixture of two *E. coli* cultures isolated from the herd; the second group was vaccinated with a commercial polyvalent vaccine and the third group consisted of the unvaccinated controls.

The sows gave birth to 642 pigs, 550 of which were early weaned (12 days) and 92 were late weaned (8 weeks). Mortality among the early-weaned pigs (25 per cent) was much higher than among the late-weaned pigs (2 per cent). The deaths were randomly distributed among the three groups which indicated that vaccination did not affect mortality.

The agglutinin titre of the colostrum of the sows was measured against living and heat-killed cells of the same two strains of *E. coli*. Blood samples were also taken from 75 pigs for the same serological tests.

Sows given the laboratory vaccine produced colostrum with a significantly higher agglutination titre than the sows given the commercial vaccine or the controls. The commercial vaccine gave a somewhat higher titre than the controls, but the difference was not always statistically significant.

The same result was obtained with sera from "early weaned" pigs, although the results were less clear cut with heat-killed antigen. The titres obtained with heat killed (O) antigens were so low that they could not be statistically analysed.

There is evidence that the increase in the agglutination titre of piglet blood is temporary and declines rapidly 3 to 4 weeks after weaning.

Vaccination resulted in higher agglutinin titres in sows' colostrum and piglets' blood, but did not affect the mortality rates. This result was probably due to the appearance of new sero-types during the experiment. It is postulated therefore that early weaned pigs are not likely to be protected against gut oedema by vaccination of the sow.

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EGG TRANSMISSION OF INFECTIOUS SYNOVITIS

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INTRODUCTION

Infectious synovitis of chickens was first reported in the United States by Wills (1954), and by Olsen, Bletner, Shelton, Munroe and Anderson (1954) working independently. The disease has since become widespread in the broiler producing areas there. In this country the disease was first seen in March 1958 (Carnaghan, 1959) and a further 7 outbreaks were diagnosed at this laboratory during the year. In 1959, the number of outbreaks diagnosed rose to 104 and the disease had spread from its original source to other broiler groups in different parts of the country. While it has mainly affected broiler chickens a few cases have been seen in chickens reared for other purposes and 2 outbreaks in adult breeding stock have been diagnosed.

Although the possibility of egg transmission of the infective agent has been realised the evidence in support of this method of transmission is slight, and there is no information as to the extent to which it occurs. Wills and Delaplane (1955) reported the development of infectious synovitis at 3 weeks of age in 1 of 1,470 progeny of breeding chickens exposed to the disease. These results were interpreted as being highly suggestive, but not conclusive evidence, of egg transmission. Thayer, Strout and Dunlop (1958) reported finding lesions in the hocks of 21-day-old chick embryos and in 1 and 6-day-old chicks from commercial hatcheries and were able to isolate the agent from a single 6-days-old chick. Snoeyenbos and Basch (1958) isolated the agent from the yolk sacs of two dead embryos from eggs laid by experimentally infected fowls. Commenting on these results the authors stated that one of the major difficulties encountered in attempting to demonstrate egg transmission of infectious synovitis under experimental conditions was to induce infection in mature chickens.

This article describes a field outbreak of infectious synovitis in point-of-lay pullets and the subsequent isolations of the infective agent from eggs laid by the flock after the clinically affected birds had been removed. Successful transmission of this agent and of another strain isolated from young chickens to adult fowls is described. The isolation of the agent from eggs laid by experimentally infected adult fowls and the development of infectious synovitis in their progeny is also reported.

HISTORY

The disease occurred in a group of approximately 600 pullets and 28 cockerels housed intensively. The birds were just over 6 months of age and were producing about 80 eggs a day when symptoms of lameness were first noticed. Two live pullets sent to the laboratory for examination were said to be representative of 70 similarly affected. The feet, hocks and wing joints were considerably

swollen due to the presence of glairy, mucoid material in the joints and tendon sheaths. Pending a definite diagnosis the owner was informed that the appearance of the lesions suggested the possibility of infectious synovitis. He thereupon examined the flock and killed 100 pullets showing swollen joints. No cockerels were affected. One month later the flock was in 50 per cent production. During this period three further birds had developed swollen joints and had been isolated. The remainder of the flock was clinically normal.

The origin of this outbreak was not established. Chicks from the same batch reared on other farms remained free of the disease. The only contact with the disease that could be found was that during the rearing period the birds had been on free range in fields approximately 200 yards from premises in which broiler chickens were known to be affected with infectious synovitis at the time. While this was considered to be a possible source of infection, experimental evidence indicates that direct contact is necessary for transmission (Wills and Delaplane, 1955).

METHODS

Arrangements were made with the owner for fifty eggs from one day's production to be sent to the laboratory weekly for four consecutive weeks for incubation. These eggs were chosen at random and represented between 15·2 and 16 per cent of the total daily production. The first batch of eggs was received approximately 3 months after the disease had first been noticed in the flock.

Eggs, chicks and adult fowls used in the experiments were obtained from a flock maintained at this laboratory which is believed to be free of Newcastle disease, pullorum disease and pathogenic P.P.L.O. infection.

Treatment of material and methods of transmission are described in the appropriate sections under "Results".

RESULTS

Isolation from Adult Fowls

Joint material from the adult birds was collected aseptically, diluted 1:5 in sterile peptone broth and 2,000 units of penicillin added per ml. Six-days-old chick embryos were inoculated with 0·4 ml. of this material into the yolk sacs and at the same time chicks between 2 and 3 weeks of age were inoculated with 0·2 ml. into the plantar pad. Examination of the joint material for bacteria and P.P.L.O. was carried out as previously described (Carnaghan, 1959).

In the first embryo passage, of the ten 6-days-old embryos inoculated, 9 were found to be contaminated by *E. coli* but one embryo which died on the 14th day after inoculation showed lesions suggestive of infectious synovitis and was bacteriologically sterile. None of the 5 chicks inoculated at the same time developed the disease. In the second embryo passage all the embryos died, 10 of them showing lesions suggestive of infectious synovitis. One of 4 chicks inoculated at the same time developed the disease. The third

egg passage produced typical lesions of infectious synovitis in all 12 embryos and in all 3 chicks inoculated with material from the previous passage.

The agent has since been maintained for 50 serial passages in embryos and has consistently produced the disease when inoculated into chicks. At the fourth passage the yolk was found to have an embryo L.D./50 titre of $10^{-4 \cdot 4}$, the end-point being calculated according to the method of Reed and Muench (1938).

Transmission to Adults

Transmission to adult fowls was carried out using yolk from the fourth and fifth serial embryo passages of the agent isolated from the field outbreak of disease in pullets (termed Strain B). The yolk was diluted 1:5 in peptone broth. Seven R.I.R. chickens aged 7 months were inoculated intravenously: four cockerels each received 4 ml. while 3 ml. was administered to the three pullets. At the same time ten 2 years-old cross-bred fowls (1 cockerel and 9 hens) were inoculated intravenously with 3 ml. A further ten 2-years-old cross-bred ensh each received 1·5 ml. of inoculum intravenously.

Ten 2-years-old cross-bred fowls (1 cockerel and 9 hens) were inoculated intravenously with 3 ml. of a 1 in 5 dilution of yolk in broth from embryos dying during the third embryo passage of the infectious synovitis agent originally isolated from broilers in this country in 1958 (Strain R). This strain had survived in yolk at —20°C. for 550 days and the infective material used was the second serial embryo passage after storage.

All 7 young adults inoculated with Strain B developed the disease. Seven days after infection 2 pullets were lame and had developed swollen hocks. Two days later the cockerels developed the disease and the remaining pullet became affected after a further 5 days. Extreme paleness of the combs and wattles developed in the sick birds and was accompanied by swelling of the hock and wing joints, profuse green diarrhoea and loss of weight. One pullet died 27 days after inoculation. The remaining birds made a slow recovery and $7\frac{1}{2}$ weeks after infection the two surviving pullets commenced laying.

In the ten 2-years-old fowls inoculated with 3 ml. the disease took a very severe course causing 7 deaths. After an incubation period of 6 days all the birds became affected with generalised swelling of the joints, profuse green diarrhoea and anaemia. Six of the 7 deaths occurred between 10 and 21 days after inoculation. The survivors commenced laying 2 months after being infected.

All ten 2-years-old fowls inoculated intravenously with 1·5 ml. of Strain B developed the disease and 2 deaths occurred. The survivors of this experiment together with those surviving the previous two experiments were penned together and the eggs were incubated weekly.

Nine of the 10 2-years-old fowls inoculated with Strain R devel-

oped infectious synovitis and three, including the cockerel died. The symptoms and lesions were similar to those produced by Strain B but the course of the disease was not as severe. The post-mortem findings were similar to those described in young birds (Wills, 1954). Bacteriological examination and examination for P.P.L.O. were negative.

The disease was successfully transmitted from infected adults to young chickens 2 to 3-weeks-old and also re-established in embryos using material from the joints and tendons as inoculum.

Isolation of the Agent from Eggs obtained from Field Outbreak

The eggs from the naturally infected pullets were incubated immediately on arrival at the laboratory in 100-egg capacity hot water incubators. The eggs were candled on the 6th day and those that were infertile were discarded. Thereafter, they were candled at 13 days and subsequently daily until the 18th day when they were left to hatch. All dead embryos were examined, cultured bacteriologically and, when sterile, the yolk or embryonic liver was harvested and stored at −20°C. until required for further use. The chicks which hatched were reared in isolation to 10 weeks of age when the majority were killed and examined. Twenty-five birds were reared to 6 months before being killed. Yolk from embryos suspected of dying from infectious synovitis and synovial material from suspected cases in chickens was inoculated into the yolk sac of 6-days-old chick embryos. A positive isolation was considered to be made when typical lesions of infectious synovitis were seen in embryos and when yolk from these embryos produced the typical disease when inoculated into the plantar pad of 2 to 3-weeks-old chicks. Generally, 3 serial embryo passages were carried out before material was considered to be negative.

Six of the first consignment of eggs were broken in transit. Six of the remaining 44 were infertile; 2 embryos died at about the 8th day of incubation and one at about the 19th day. Although this embryo showed lesions suggesting infectious synovitis it was not possible to isolate the agent. Thirty-five chicks hatched but none developed the disease or showed any lesions when killed at ten weeks of age.

The second batch of eggs consisted of 49, of which 2 were infertile. Twelve embryos died during the second week of incubation and one during the third week. There were 8 dead-in-shell and 26 hatched. Three of these were weak chicks and died within a few days of hatching.

The agent causing infectious synovitis was isolated from the pooled yolks of 3 embryos which died during the second week of incubation and from one dead-in-shell embryo. One chick developed lesions of infectious synovitis at 7 days of age and was isolated from the remainder. It was killed when five weeks old and the infective agent was isolated. The remaining chicks reared satisfactorily and were normal when killed at 10 weeks of age.

There were 4 infertiles in the third batch of 50 eggs, 11 died during incubation and there were 9 dead-in-shell. Twenty-six chicks hatched. The agent was isolated from the pooled yolks of 3 embryos which died on the 15th day of incubation. These embryos showed identical lesions to those seen in experimentally infected embryos. The disease did not develop in any of the chicks which hatched.

In the fourth batch of 50 eggs, 6 were infertile, 7 embryos died during incubation and there were five dead-in-shell. Thirty-two chicks were hatched. Two embryos which died on the fourteenth and fifteenth days of incubation had typical lesions of infectious synovitis and the causal agent was recovered from both. None of the chicks developed the disease.

Isolation of the Agent from Embryos and Chicks of Eggs from Experimentally Infected Adults

The procedure outlined in the preceding section was adopted for the eggs laid by the survivors of the experimental infection in adult fowls with strain B. Altogether 12 hens and pullets and 2 cockerels survived the disease and these were placed together in a pen two months post infection. Eggs were incubated weekly in hot water incubators and the same procedure followed as for the eggs received from the field outbreak of infectious synovitis. The chicks were reared to 6 months of age in isolation units before being killed. A control group of 120 R.I.R. chicks from the laboratory farm was reared in similar accommodation.

Chicks which developed infectious synovitis were removed from the group at the first sign of the disease in order to reduce the possibility of transmission by contact.

During a period of 32 weeks a total of 463 eggs was incubated of which 158 were infertile. Two hundred and twelve chicks hatched of which 181 survived to 8 weeks of age and were reared to 6 months.

The agent was isolated from the pooled dead embryos of 5 of the 32 weekly incubations, the last isolation being approximately 24 weeks after experimental infection of the parent stock. Other isolations were made from eggs laid 14, 16, 18 and 23 weeks after infection. Five cases of infectious synovitis occurred in the progeny. These were in chicks hatched from eggs laid 17, 20, 21, 22 and 24 weeks after infection. The age at which the first signs of the disease was noticed varied from 75 to 98 days, the average being 80 days. None of the control birds developed the disease.

When the adult fowls were killed 343 days after they had been infected all but one were found to have chronic lesions of infectious synovitis and the agent was recovered from the pooled synovial material by inoculation into the yolk sacs of six-days-old chick embryos. Typical lesions were produced on the third serial passage and yolk from this passage reproduced the disease when inoculated into chicks.

DISCUSSION

The results of these experiments show that both under field and laboratory conditions egg transmission of the causal agent of infectious synovitis occurs. In the natural outbreak of disease approximately 6 per cent of 171 fertile eggs selected at random from a single day's production each week for 4 consecutive weeks were found to be infected, as judged by the number of dead embryos showing lesions of infectious synovitis and cases of disease in hatched chicks, and by the subsequent isolation of the agent. By the same criteria only 3 per cent of 305 fertile eggs laid by experimentally infected fowls were infected, but this was over a period of 32 weeks. The infected eggs from the experimental birds were all laid between 14 and 24 weeks after infection. It was not determined whether these were laid by one or more of the infected hens, nor whether transmission takes place by transovarian means or by penetration through the egg shell.

The disease was readily transmitted to adult fowls of various ages in the laboratory with strains of the agent isolated from both adult and young chickens. The facility with which it was induced in mature fowls was surprising in view of the difficulty encountered by Snoeyenbos and Basch (1958) in their attempts. The successful transmissions were accomplished on several occasions during severe weather in October and November and as subsequent attempts have not been so successful work is in progress to determine whether seasonal conditions have any bearing on the susceptibility of adult fowls to the infectious synovitis agent.

The progeny of the artificially infected fowls developed the disease at a later age than is usually seen in natural outbreaks of the disease. It was not established whether this was due to any specific feature of the strain of the agent or whether it was because the birds were reared in conditions which were not comparable to those existing in broiler houses.

The recovery of the infective agent from synovial material 343 days after infection indicates that it remains viable for long periods in infected joints and tendon sheaths. Benton and Cover (1959) found that tissues from chronically affected birds failed to infect susceptible birds and assumed either complete absence or low infectiousness of the agent. The results reported here indicate that the former alternative is incorrect and the fact that it was isolated from embryos 23 weeks after infection of the parent stock suggests that it may remain at an infectious level for longer than has been previously realised.

CONCLUSIONS

An account is given of an outbreak of infectious synovitis in laying pullets and the subsequent isolation of the infective agent from 6 per cent of embryos and a single chick hatched from an egg produced by the clinically normal survivors of the disease.

The disease was successfully transmitted to adult fowls in the laboratory and the causal agent was isolated from embryos dying during incubation and from chicks hatched from eggs produced by the survivors of the experimental disease.

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QUANTITATIVE STUDIES OF *MYCOBACTERIUM JOHNEI* IN THE TISSUES OF SHEEP

I. Routes of Infection and Assay of Viable *M. johnei*

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INTRODUCTION

Control of Johne's disease by vaccination was first suggested by Vallée and Rinjard (1926) while Vallée, Rinjard and Vallée (1934) claimed that their method had proved effective in France. Since 1946 vaccination has been under trial in Great Britain in selected infected herds of cattle free from tuberculosis (Control of Johne's Disease Project No. 207 O.E.E.C. 1956). Sigurdsson (1960) also has reported excellent control of Johne's disease in sheep in Iceland, using heat-killed *M. johnei* in oil as vaccine.

If vaccination can reduce the incidence of Johne's disease, it would be useful to have a method by which vaccines could be assayed without recourse to the experimental production of clinical disease. Apart from the obvious saving in time, a more sensitive technique might also permit investigations to be made of the epidemiology of the preclinical stages of infection with *M. johnei*. These investigations were carried out in sheep because sheep are naturally susceptible to Johne's disease.

In the present preliminary experiments we have used techniques which attempted the quantitative assay of viable *M. johnei* in the tissues after challenge with known numbers of bacilli by the intravenous and oral routes. Since sheep were more satisfactory for research than small laboratory animals and less expensive than cattle, in the preliminary work the investigations were conducted in non-vaccinated and vaccinated sheep in parallel. As these experiments extended over a period of years and each experiment covered many months, it is more convenient to describe the investigations in non-vaccinated and vaccinated animals separately. This paper describes in detail the techniques of quantitative assay of *M. johnei* in the tissues of unvaccinated sheep. The comparative results obtained by these techniques in vaccinated and non-vaccinated animals will be described in another publication (Brotherston, Gilmour and Samuel, 1961).

MATERIALS AND METHODS

Experimental animals. The experimental sheep were South Country Cheviots of either sex, born from apparently healthy cast ewes which had been over-wintered at the Institute. When required, the ewes and lambs were allocated at random to the various groups and housed in pens in a covered yard. All sheep were fed a ration of concentrates, hay and

water ad lib. Some lambs were used before weaning at 3 weeks of age; others were retained until after weaning and used at 12 weeks of age or older. Untreated animals were introduced into each group during dosing to determine the level of cross infection. Further control lambs were introduced 1 month after dosing had ceased to determine if cross-infection occurred within some of the experiments. At this time infected animals were moved to clean pens to avoid contact with faeces excreted during the dosing period.

Random samples of challenged and control animals from the experimental groups were killed and examined at intervals between 1 month and 22 months after the last infecting dose.

Mycobacterium johnei *and culture media.* The strains of *M. johnei* used were 101, originally recovered from a clinical case of the disease in a sheep and VB/4 from a clinically affected cow. The second subculture of strain 101 and the fourth subculture of strain VB/4 were used to provide the inocula. Both isolates had the cultural and biological characters of *M. johnei var. bovis,* in that the "phlei factor" was essential for growth. Rough, yellowish-white colonies were easily visible on solid medium at 4 to 6 weeks but not before and the organisms were non-pathogenic for mice, guinea-pigs, rabbits and fowls. The media were modifications of the Dubos solid and liquid media described by Williams Smith (1953). To control contamination when carrying out viable counts on the mucous membrane of the intestine, the solid medium was further modified to include Actidione* and chloramphenicol at a concentration of 200 and 50 μg. per ml. of medium respectively. The solid medium was dispensed in 6·25 ml. quantities and sloped in 1 oz. McCartney bottles. It was excellent for both primary isolation and for quantitative work, particularly as it was translucent and colonies could be counted with ease. Before inoculating slopes, the water of condensation was drained and the medium allowed to dry at room temperature (60 to 65°F) for 48 hours with cotton wool plugs. When the slopes were properly dried the seeding inoculum of 0·1 ml. was absorbed and there was no development of secondary colonies by splash or droplet during subsequent handling and incubation.

Preparation of infecting inoculum. Suspensions were made by grinding in a small Griffith tube, 6 to 8-week old growth from solid medium, using as a diluent liquid Dubos medium. This was allowed to stand for 20 minutes. The supernate with the addition of more medium was reground in another tube and then stood for a further 20 minutes. From this supernate, a suspension equivalent to Brown's scale 2 was prepared by comparison with a ground-glass standard in an EEL nephelometer†. Microscopically the suspension contained single bacilli or small clumps with rarely more than 10 bacilli per clump. In these preliminary experiments we wished to have some measure of the number of viable units of *M. johnei* present in a suspension which could be reproduced within reasonable limits on any occasion by adopting the method just described. The viable counting technique which will be described in detail later was applied to the standard suspension of Brown's scale 2 and it was found that the counts varied between 10^8 and 10^9 viable units per ml. We have tried to replace the method of describing doses of living *M. johnei* in terms of wet weight with one which gave an indication of the number of viable

*L. Light & Co. Ltd., Colnbrook, England.

†Evans Electroselenium Ltd., Harlow, Essex.

units, but as a basis of comparison it may be of interest to note that a suspension of Brown's scale 2 contains about 2 mg. wet weight of bacilli per ml.

Viable counts in the mesenteric lymph nodes. At the end of each experimental period animals were slaughtered, bled and skinned, the abdomen being swabbed with boiling water before being opened. The ileo-caecal, posterior mesenteric, mesenteric, portal, pharyngeal lymph nodes, spleen and portions of liver were removed with sterile precautions and weighed. These tissues were either examined separately or groups of nodes such as the mesenteric nodes were harvested, pooled and emulsified. From these suspensions the mean viable count per lymph node or per gramme of the pooled tissues was calculated as follows. Between 20 and 50 g. of lymph nodes were collected and after mincing, samples of about 4 g. were removed to prepare a 20 per cent homogenate. The whole portal node or group of nodes were removed for examination. At least 5 g. was taken from different sites of the liver and spleen and treated similarly. The samples were ground in a Griffith tube or homogenised with a stainless steel masticator** attached to a universal container, in liquid medium to make a 20 per cent w/v emulsion. One tenth ml. volumes of the 20 per cent homogenates and of 10-fold dilutions of 1 ml. samples in liquid medium at 10-fold, 100-fold and 1,000-fold, were deposited on the dry surface of a slope of solid medium. The slopes were allowed to remain horizontal on the bench for 24 hours and next day the cotton wool plugs were replaced with screw-caps and the containers incubated upright. Counts of colonies were made after 8 and again after 12 weeks' incubation at 37°C. The number of viable units of *M. johnei* for each tissue was calculated by multiplying the mean of the numbers of colonies on replicate slopes inoculated with the same dilutions by the appropriate dilution and sampling factors. Using the method described, it was not possible to detect fewer than 50 viable units or $1 \cdot 7$ $\log_{10}$ viable units of *M. johnei* per g. of lymph node tissue. Slopes having from 10 to 100 colonies were counted preferentially, but in many instances it was necessary to calculate from fewer than 10 colonies per slope. In the early experiments when dealing with individual lymph nodes and those tissues which were not treated with oxalic acid to control contamination, one aliquot was taken from each homogenate and each dilution was assayed in triplicate, in order to allow for possible contamination and to conserve media. There was little variation between the replicate counts of the dilutions, so in later experiments the procedure of carrying out viable counts on several aliquots of the tissue homogenates was adopted. This is described in the next paragraph.

Viable counts in the mucous membrane of the small intestine. The intestines were removed from the abdomen after samples of the mesenteric lymph nodes or other tissues had been collected aseptically. The duodenum, ileum and jejunum were separated from their mesenteric attachments and their contents expelled. The mucosa was exposed by slitting the intestinal wall with bowel scissors. The intestinal wall from the pylorus to the ileo-caecal valve was divided into three equal lengths. Each length of about 20 feet was treated separately as follows: The length of intestine was washed in tap-water to remove faecal debris and the excess water drained. The intestines were stretched on a hard flat surface. The mucosal and

**Measuring and Scientific Equipment Ltd., England.

muscular coats were separated from the serosa by scraping the mucosal side of the serous coat of the intestinal wall with a glass slide. Histopathological examination of the serous coat left after this operation revealed that the mucous and submucous coats were removed efficiently. The volume of mucosa removed was measured, made up to 200 ml. with sterile distilled water and homogenised for 2 minutes in an Atomix†† container. Four 10 ml. aliquots of the homogenate were measured into sterile 4 oz. medicine flat bottles and 10 ml. of a 10 per cent oxalic acid solution were added to each. The mixtures were placed in an incubator at 37°C for 30 minutes after which 10 ml. of sterile distilled water were added to each. The homogenates were shaken vigorously after each addition of fluid and at intervals during incubation. The final dilution of oxalic acid in the homogenates was 2/3 of 5 per cent. One tenth ml. volumes of the 4 oxalated aliquots of each homogenate and of 10-fold dilutions of 1 ml. samples of them in liquid medium were sown on to solid medium and treated as described previously. Using these methods it was not possible to detect fewer than 1,500 viable units or $3{\cdot}18$ $\log_{10}$ viable units of *M. johnei* per 20 ft. length of intestine. It was observed that irregular results were obtained from counts of undiluted homogenate (which contained 2/3 of 5 per cent oxalic acid) due to the inhibitory effects of either residual oxalic acid or cell debris. At subsequent examinations, therefore, the lowest dilution inoculated was 10^{-1} and the smallest detectable number of viable units was 15,000 or $4{\cdot}18$ $\log_{10}$ viable units. It must be emphasised that where the irregular counts were observed, this did not bias the results recorded in the experiments, as in these instances the total counts were based on 10-fold and 100-fold dilutions of the oxalated homogenate. There was little variation between the counts from the 4 aliquots of each suspension which was a tribute to the efficient distribution of the bacilli throughout the bulk of the material after homogenisation. The number of viable units of *M. johnei* for each length of intestine was calculated by multiplying the mean count by the appropriate dilution and sampling factors. The calculated viable counts for each length of intestinal mucous membrane were added to obtain the total viable count of the small intestine.

RESULTS

Survival in Lymph Nodes of Sheep after Intravenous Infection (Exp. I)

In this experiment 32 lambs with their ewes were run together. They were weaned at 10 weeks old and divided into two groups of 16 untreated control lambs (Group 1) and 16 lambs infected at 10 weeks old (Group 2). The infected animals were injected intravenously with 2 ml. of a 10-fold dilution of a standard suspension of *M. johnei*, containing about 10^7 viable units of bacilli. Lambs from each group were killed and examined at intervals of 3 to 4 months, 6 to 7 months, 8 to 9 months and 21 to 22 months after infection. By this means it was hoped to obtain information about the "lag phase" and the progress of infection. At each of these times the viable unit counts of *M. johnei* were calculated in the ileo-caecal, the posterior mesenteric and the portal lymph nodes. The reason for selecting these tissues will be discussed later.

††Measuring and Scientific Equipment Ltd., England.

The viable unit counts of *M. johnei* in the tissues, expressed as $\log_{10}$ units per whole node are shown in Table 1. There was no evidence of infection in any of the undosed control sheep examined at the same times as those injected with *M. johnei*. At 3 to 4 months, sheep 3548 and 3542 showed heavy colonisation of the portal and both mesenteric nodes so that the viable counts could only be estimated to be over 5 $\log_{10}$ units. When examined at 6 to 7 months, all 4 animals showed persistence of *M. johnei* but there was a marked variation in the sites at which bacilli could be detected. For example, in sheep 3543 the viable count in the portal node was 5·4 units, but

TABLE 1

LOG_{10} VIABLE UNITS OF *M. JOHNEI* PER WHOLE NODE

Portal, posterior mesenteric and ileo-caecal lymph nodes after intravenous infection with 10^7 viable units

Time in months	*No.*	*Portal node*	*Posterior mesenteric node*	*Ileo-caecal node*
3/4	3548	>5·0	>5·0	>5·0
	3542	>5·0	>5·0	>5·0
6/7	3543	5·4	—	—
	3441	—	3·1	3·3
	3554	4·1	2·3	4·6
	3555	1·7	3·5	—
8/9	3557	—	—	—
	3549	—	3·8	3·1
	3553	2·0	—	—
	3551	2·0	3·4	3·4
21/22	3540	—	—	—
	3545	—	3·5	—
	3546	—	3·0	2·1
	3544	—	—	2·7
	3550	—	—	1·8
	3552	—	—	—

— = less than 1·7 $\log_{10}$ units.

Untreated control animals had no detectable infection at any time.

the other sites were free or infected below the detectable level. At 8 to 9 months, only 1 sheep (3557) was apparently free in all sites, while the others had detectable bacilli in one or other of the tissues examined. The remaining 6 animals were examined at 21 to 22 months after challenge and *M. johnei* was no longer detectable in the portal lymph nodes of any, but in 4 persistence of *M. johnei* was demonstrated in one or other of the intestinal lymph nodes. Viable bacilli were not detected at all in 2 of the sheep.

Occurrence in the Lymph Nodes and Tissues of Sheep after Oral Infection (Exp. 2)

A small flock of cast Cheviot ewes and lambs was divided into 2

groups which were kept apart. The lambs were treated as follows and weaned at 10 weeks old.

Group 1. Fourteen lambs not older than 7 days were dosed with a single suspension which contained about 10^8 viable units. Two lambs were given this dose on 3 occasions at intervals of 1 week. Six lambs were left undosed.

Group 2. A group of 10 lambs about 7 to 10 days old were dosed once weekly over a period of 8 weeks with a suspension, each deos containing about 10^8 viable units of bacilli. Four untreated lambs were allowed to run with this group commencing 1 week after the completion of dosing.

Pairs of lambs from each group were examined at intervals between 1 week and 53 weeks after the first dose was given. An undosed in-contact animal was also examined at each period starting at 4 weeks in Group 1 and 16 weeks in Group 2. On each occasion the viable counts of *M. johnei* were calculated per g. of (a) the posterior mesenteric group of nodes; (b) the mesenteric nodes; (c) the portal nodes; (d) the liver and (e) the spleen, as described previously. By this means it was planned to observe the fate of *M. johnei* reaching the lymph nodes associated with the intestine or the body organs at times between 1 week and 1 year after oral dosing. Material for histological examination was taken by removing at least 2 lengths of intestine about 1½ inches long and including a Peyer's patch at random from the duodenum, the jejunum and the terminal iluem. Additional lengths of intestine were removed if macroscopic changes were obvious.

Group 1. The viable counts in the tissues of animals expressed as $\log_{10}$ units per g. are shown in Table 2. The lambs given an oral dose of about 10^8 viable units had no detectable level of infection in any of the organs examined 1 week later. Similarly those given this dose on 3 occasions at intervals of 1 week were negative on examination 1 week after the last dose. Subsequent examinations refer only to lambs dosed once. At 4 weeks after infection 1 of 2 lambs examined had detectable infection of the mesenteric group of nodes, but of no other tissues. At examinations between 8 weeks and 52 weeks after infection, the presence of viable units was easily detected in the lymph nodes associated with the intestine. Serial examination, however, did not reveal a tendency for a progressive increase with time of the viable counts in the lymph nodes. *M. johnei* were not detected in the spleen, liver or portal nodes on any occasion. Untreated control animals proved to be uninfected at each examination.

Group 2. The results of examinations are shown in Table 3. The first animals were 2 lambs of about 10 weeks old. Viable unit counts in the mesenteric lymph nodes were of the same order as in animals dosed once in Group 1 and examined at 8 weeks. Of the 2 sheep examined at 16 weeks 4990 had 5·2 viable units in the posterior mesenteric nodes and bacilli were detectable in the portal node, but

TABLE 2

OCCURRENCE OF *M. JOHNEI* IN LYMPH NODES OR TISSUES
Lambs dosed orally with 10^8 viable units

Weeks after dosing	*No.*	*Log_{10} viable units per g.*		
		Posterior mesenteric	*Mesenteric*	*Portal Liver Spleen*
1	166	—	—	—
1	138	—	—	—
4	140*	—	—	—
4	144*	—	—	—
4	159	—	—	—
4	153	—	2·6	—
8	4969	3·5	—	—
8	4988	3·5	2·9	—
16	4954	3·5	2·6	—
16	4991	3·7	3·9	—
24	4967	3·9	4·1	—
24	4974	3·0	2·9	—
32	4971	2·9	3·4	—
32	4976	2·6	2·9	—
52	4992	4·1	4·0	—
52	4973	—	2·6	—

*Nos. 140 and 144 received 10^8 viable units per week for 3 weeks and were examined 1 week later.

— Less than $\log_{10}$ 1·7 viable units.

not in the liver. In subsequent examinations at 24 and 32 weeks, there was no evidence of a progressive increase in the viable counts as a result of the greater dose given to the Group 2 sheep. There was only 1 animal remaining at 53 weeks and this had become an early clinical case of Johne's disease with counts of over 5 viable units per g. in both mesenteric lymph nodes. There was no detectable infection in the portal node, liver or spleen.

Occurrence in the Small Intestine and associated Lymph Nodes of Lambs and Sheep after Oral Dosing (Exp. 3)

Groups of 3-week old lambs each made up of 12 animals, were dosed orally, once weekly for 10 weeks, with 1 ml. of the standard suspension of strain BT 2, 1 ml. diluted 10^{-3} and 1 ml. diluted 10^{-6}. The standard suspensions, made from 6 to 8 week old cultures of strain

TABLE 3

OCCURRENCE OF *M. JOHNEI* IN LYMPH NODES OR TISSUES
Lambs dosed orally with 10^8 viable units per week for eight weeks

Weeks after first dose	*No.*	*Log_{10} viable units per g.*		
		Posterior mesenteric	*Mesenteric*	*Portal Spleen Liver*
10	152	3·2	2·6	—
10	4959	3·6	3·8	—
16	4990	5·2	3·6	portal 2·6
16	4977	—	2·6	—
24	4972	—	2·6	—
24	4979	—	2·6	—
32	4964	—	2·6	—
32	4980	3·0	3·6	—
53†	4962	5·3	5·1	—
53	4987	Died other causes		

One untreated control animal was killed at each time starting at 16 weeks. One showed detectable infection in the mesenteric nodes at 24 weeks (2·6 viable units per g.); the others remained free.

†Clinical case.

— Less than $\log_{10}$ 1·7 viable units.

101/2, contained at the 1st, 5th and last dose 2×10^8, 9×10^7 and 3×10^8 viable units per ml. respectively. The lambs were judged to have been given a total dose of viable organisms at the end of 10 weeks as follows:

Group 1, at least 10×10^2 (about 10^3 viable units); Group 2, at least 10×10^5 (about 10^6 units); Group 3, at least 10×10^8 (about 10^9 units).

Two untreated lambs were placed in contact with each of the above groups for the duration of the dosing period and until they were killed at the first examination of each group. Three additional untreated control lambs were then introduced into each group.

Unselected samples of infected and control lambs from each group were killed and examined 1, 4 to 5 and 8 to 9 months after the last infecting dose. The retropharyngeal nodes were also removed and cultured after treatment with oxalic acid for the presence or absence of *M. johnei*. Material for histopathological examination was taken from 6 sites at about 10 foot intervals from the pylorus to the ileo-caecal valve. Each block contained an area of lymphoid tissue.

The viable counts from the intestinal mucosa, the mesenteric lymph nodes and the retropharyngeal nodes of lambs dosed orally

TABLE 4

VIABLE COUNTS OF *M. JOHNEI* IN THE INTESTINAL MUCOSA AND MESENTERIC LYMPH NODES

Lambs infected orally

Time after infection	*Group 1. Dose 3 $\log_{10}$ units*			*Group 2. Dose 6 $\log_{10}$ units*			*Group 3. Dose 9 $\log_{10}$ units*		
	10 ml. BT2 × 10^{-6}			*10 ml. BT2 × 10^{-3}*			*10 ml. BT2*		
	Sheep	*Mesenteric node/g.*	*Small intestine*	*Sheep*	*Mesenteric node/g.*	*Small intestine*	*Sheep*	*Mesenteric node/g.*	*Small intestine*
1 month	6215 C	—	—	6202 C	—	—	6228 C	—	—
	6216 C	—	—	6201 C	—	—	6229 C	2·60	5·68
	6204	2·74	4·97	6160	2·70	5·35	6225	2·51	4·78
	6203	2·30	5·29	6162	3·74	5·67	6226	3·65	5·18
	6206	2·74	5·38	6161	—	5·82	6224	4·07 R	5·96
	6205	3·18	6·34	6163	—	6·36	6223 (clinical)	5·40 R	8·02
4–5 months	6703 C	—	—	6249 C	—	—	6271 C	—	—
	6209	—	—	6166	—	—	6221	—	—
	6214	—	—	6169	—	—	6227	—	5·18
	6208	—	—	6170	—	—	6222	2·89	6·66
	6211	—	—	6167	1·70	4·18	Died earlier		
8–9 months	6258 C	—	—	6153 C	—	—	6256 C	—	5·32
	6285 C	—	—	6146 C	—	—	6284 C	3·69 R	6·90
	6207	—	—	6164	—	—	6217	—	—
	6213	—	—	6165	—	—	6218	—	4·13
	6212	1·7o	—	6168	—	—	6219	3·49	8·12
	6210	—	3·48	6171	—	4·65	6220 (clinical)	>8·0 R	>8·0

C = untreated control lamb.

R = retropharyngeal node +ve.

At 1 month and 8 to 9 months — = less than 1·7 $\log_{10}$ units per g. and 3·18 $\log_{10}$ units per length of intestine.

At 4 to 5 months — = less than 1·7 $\log_{10}$ units per g. and 4·18 $\log_{10}$ units per length of intestine.

with 3 dose levels of *M. johnei* and killed at 1 month, 4 to 5 months and 8 to 9 months after infection are shown in Table 4. In Group 1 at 1 month, the individual counts in the mesenteric nodes varied between 2·3 $\log_{10}$ viable units and 3·1 units per g. In the intestine the counts varied from 4·9 units to 6·3 units. Retropharyngeal nodes were not shown to be infected. At 4 to 5 months after the last dose, infection had dropped below the detectable level in all the animals and at all sites examined. Between 8 to 9 months, only 2 animals were infected, 6212 with 1·7 units per g. in the mesenteric nodes only and 6210 with 3·4 units in the intestine only. Retropharyngeal nodes were uninfected.

In Group 2, at 1 month, all the sheep were infected at about the same level as those in Group 1. The exceptions were 6161 and 6163 in which no *M. johnei* were detected in the mesenteric nodes. The retropharyngeal nodes of all the animals were free of infection. At 4 to 5 months after dosing only 1 animal, 6167, had viable counts in the mesenteric nodes and the intestine and these were below the levels found at the earlier examination. At 8 to 9 months, only one animal, 6171, had detectable infection in the intestine only.

In Group 3, which received 1,000 times the dose of Group 2 and 1,000,000 times the dose of Group 1, the results differed from those obtained in Groups 1 and 2. At 1 month after dosing the counts in the mesenteric nodes varied from 2·5 $\log_{10}$ units per g. to 5·4 units and in the intestine from 4·78 to 8·02 units. While these were not markedly higher than in the other groups, 6223 was a clinical case of Johne's disease and 6223 and 6224 showed infection of the retropharyngeal nodes. In addition one control animal 6229 had a high level of infection in the mesenteric nodes and the intestine. At 4 to 5 months, 2 animals out of 3 were infected, 1 sheep having died earlier in the experiment from other causes. In 6222 both mesenteric nodes and the intestine were infected, the counts being 2·8 units and 6·6 units respectively, while 6227 had 5·18 units in the intestine and *M. johnei* was not detectable in the mesenteric nodes. The control animal was not infected on this occasion. At 8 to 9 months, 3 out of 4 animals were infected. No. 6218 had 4·1 units in the intestine only, 6219 had 3·49 units per g. in the mesenteric nodes and 8·12 units in the intestine, while 6220 was a clinical case in which the viable count was too high to be determined at the dilutions used. The retropharyngeal nodes were infected in this case. In addition both contact control sheep were infected. No. 6256 had 5·3 units in the intestine only; 6284 had 3·69 and 6·9 units in the mesenteric nodes and intestine respectively. The retropharyngeal nodes were also infected.

DISCUSSION

There is little known about preclinical infection in Johne's disease, particularly in respect of the sites of primary lesions, the predilection sites of multiplication of the organisms and the "lag

phase" between primary infection and the advent of the classical syndrome of clinical disease. The preliminary steps in this investigation were to determine the dose of *M. johnei* which would infect most of the animals used, the route by which the dose should be given and the time after infection when the animals should be examined.

McEwen and Samuel (1958) had already noted that after intravenous infection, viable *M. johnei* were most frequently recovered from the lymph nodes draining the liver and the last part of the small intestine. We chose the portal, the posterior mesenteric and the ileo-caecal lymph nodes in which to compare the survival of *M. johnei* in sheep examined after intravenous challenge. An equal number of unchallenged control animals were examined at the same times and cultural or postmortem evidence of infection with *M. johnei* was never found. It was reasonable to assume therefore that the viable bacilli recovered from the experimental sheep were there solely as a result of the challenge dose and not as a result of contact infection. At 3 to 4 months after challenge large numbers of viable bacilli were recovered from the portal and the mesenteric lymph nodes and most of the sheep were infected at one site or another when examined at later periods. At 21 to 22 months 2 animals had no detectable infection at all; in 4, *M. johnei* was still present in the mesenteric lymph nodes, but in all 6 sheep bacilli were not detectable in the portal lymph nodes. The over-all results suggested that *M. johnei* introduced by the intravenous dose were distributed in the tissues where they survived, but the numbers of bacilli in the tissues were reduced the longer animals were allowed to survive. If this were so then about 10^7 viable units of *M. johnei* intravenously should be a reasonable dose with which to test the resistance of animals and not one which would overwhelm any immunity likely to be stimulated by vaccination. It is clear that the bacilli survived in the mesenteric nodes of most sheep for almost a year however. It follows that either the immunity stimulated in the body by the presence of viable *M. johnei* was of a low grade insufficient to overcome the original infection or else the *M. johnei* in the mesenteric lymph nodes were there also as a result of an invasion from unrecognised foci of infection, probably in the intestinal mucous membrane. This will be discussed in relation to the subsequent experiments following infection by the oral route.

Groups of lambs were given single and repeated oral doses of *M. johnei* in order to ensure that one group was challenged under conditions approaching those to which lambs might be exposed in an infected environment. Untreated control animals were exposed with the infected lambs, but since infection was detected in only 1 such animal in the group which received repeated doses it can be assumed that contact infection did not play an important part in the experimental results which have been recorded. After a single dose of viable bacilli or in respect of lambs 140 and 144 given 3 doses at weekly intervals, *M. johnei* was established in the nodes associated

with the intestine in only 1 animal out of 6 examined between 1 and 4 weeks. From 8 weeks onwards, bacilli were detected in all animals, but there was no evidence of progressive infection in the lymph nodes associated with the intestine. *M. johnei* was never found in portal lymph nodes, the liver or the spleen. A very similar situation developed in lambs which had received repeated doses and were examined at 10 weeks to 1 year after the first dose. Although histopathology of the intestine will be described in detail elsewhere, briefly, the findings were that microscopic lesions were not detectable in the intestine until after 4 weeks following oral dosage. The appearance of these lesions in the intestine seemed to coincide with the time at which viable *M. johnei* were detectable in the lymph nodes associated with the intestine. Microscopic lesions were found in the intestines of most of the animals killed between 4 weeks and 1 year after infection.

This suggests that the following tentative conclusion can be drawn about the relative merits for epidemiological investigations of the two routes of challenge. The examination of the tissues after intravenous or oral infection did not suggest that the predilection site of multiplication of *M. johnei* was the liver, spleen or the lymph nodes associated with the intestine. On the other hand the evidence shows that after oral dosing *M. johnei* was confined to the lymph nodes associated with the intestine and that the bacilli could be multiplying unrecognised in the mucous membrane of the gut. In this latter event, an examination of the mesenteric lymph nodes would provide only an indirect measure of the activity of *M. johnei* in the intestine. It is possible also that vaccination might enable animals to prevent or limit the invasion of the lymph nodes by *M. johnei* from the intestine and bacilli which reached the tissues via the blood would certainly confuse quantitative cultural studies by reason of their ability to survive for long periods. It was decided therefore to use the oral route of infection and by developing a technique for the viable count of *M. johnei* in the mucous membrane of the intestine itself, to investigate the problem more fully.

The following inferences have been drawn from the experiments on oral dosing with increasing numbers of viable units of *M. johnei* of lambs killed at different times after the last challenge dose. With a small challenge dose of about 10^3 viable units, *M. johnei* could be shown to multiply 100- to 1,000-fold in the mucous membrane of the intestine. The increase occurred comparatively quickly after challenge and was observable at 1 month after the last dose. When lambs were examined at later periods, however, the infection had fallen below the level detectable by the technique used. Increasing the challenge dose 1,000 times did not decrease the chances of spontaneous recovery in lambs examined up to 9 months later. When the challenge dose was increased to about 10^9 viable units however, the chance of establishing progressive infections and clinical disease in lambs was greatly increased. A clinical case of Johne's disease

occurred as early as 1 month after the last infecting dose and also at 8 to 9 months. Infection was detectable in the retropharyngeal lymph nodes of 4 animals. One control lamb in contact with Group 3 became infected after 1 month and 2 control animals were infected after 7 to 8 months in contact. These findings are in marked contrast to those observed in the other groups and indicate that a more active form of disease had been established in Group 3. There appeared to be little difference in the numbers of viable units of *M. johnei* recovered from the small intestine or the lymph nodes except where animals became clinical cases. In these, the viable counts were increased from 100- to 1,000-fold higher than in other animals. Histopathological examination showed that more extensive lesions were to be found in all the sheep given the largest dose. These lesions cannot be solely a result of multiplication of *M. johnei* since the counts in the preclinical cases of Group 3 were not markedly different from the infected sheep in Groups 1 and 2.

There was considerable variation in the counts obtained from the 3 lengths of the small intestine of individual sheep which were examined in these studies. Counts were not consistently greater in any particular part of the intestine and since microbial contamination very seldom interfered with the cultures this was not responsible for any variation. After oral dosing infection seemed to be equally possible in any part of the mucous membrane between the pylorus and the ileo-caecal valve. Histopathological examination indicates that those areas of intestine with the greatest concentration of lymphoid tissue, that is the Peyer's patches of the duodenum, jejunum and ileum and the last part of the ileum adjacent to the ileo-caecal junction, are the sites at which *M. johnei* were mainly concentrated. The colon and caecum were not examined, but it would be surprising if conflicting results were obtained between the upper and lower parts of the intestine in the same animal at those early stages of the disease.

Evidence has been presented to show that the intestine is the most likely site for multiplication of *M. johnei*, but progressive infection cannot be demonstrated in the preclinical stages of the disease by an examination confined to the body tissues or lymph nodes associated with the intestine. It was also shown that examination of the lymph nodes alone would have missed a number of animals which were infected. Except in the first experiment these findings were obtained as a result of oral dosing and we have not studied the occurrence of *M. johnei* in the intestine as a result of intravenous dosing. Before using the intravenous route in future studies on epidemiology or immunity it would be necessary to examine the relationship of the size of dose to the occurrence of *M. johnei* in the intestine. It would seem that whichever route was selected, the techniques which we have described were useful in elucidating the preclinical stages and early host response to infection with *M. johnei*. The reaction of the host with reference to the amount

and frequency of infection and their significance in the production of clinical disease requires further investigation.

CONCLUSIONS

In lambs given *M. johnei* by the intravenous and oral routes progressive infections could not be detected by quantitative viable counts of the liver, spleen or the mesenteric lymph nodes.

By the oral administration of graded doses of *M. johnei* it was possible to show that the intestinal mucosa was the most probable site of multiplication. Only by comparing viable unit counts from the intestinal mucosa at 1 month after the last dose could progressive infection be demonstrated in the intestine. In the majority of animals, increasing the infective oral dose by factors of 10^3 and 10^6 did not result in an increased recovery of *M. johnei* from the intestine or associated lymph nodes. The exceptions were the animals which became obvious clinical cases of Johne's disease. Clinical cases occurred in the group receiving the largest oral dose, and there was an apparent correlation between the size of the dose and the severity of the disease produced. In critical studies on infection and resistance in Johne's disease it will be necessary to examine quantitatively the occurrence of *M. johnei* in the intestine as well as in the associated lymph nodes.

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QUANTITATIVE STUDIES OF *MYCOBACTERIUM JOHNEI* IN THE TISSUES OF SHEEP

II. Protection Afforded by Dead Vaccines

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INTRODUCTION

There is evidence to suggest that immunity to tuberculosis may operate by restricting multiplication of the tubercle bacillus in the body rather than by total destruction of the organism, and that resistance can be better expressed by comparing the numbers of bacilli in the tissues of vaccinated and unvaccinated mice after challenge than by the prolonged survival time of individuals within groups (Dubos, Shaefer and Pierce, 1953). In Johne's disease a similar method would have special merit if it could detect low-grade levels of immunity and overcome the delay occasioned by the apparent long incubation period following infection of susceptible experimental animals. In previous experiments (Brotherston, 1957-58) it was shown that some advantage was conferred to guinea-pigs and mice by experience with heat-killed antigens of *M. johnei* before infection in that numbers of viable *M. johnei* in the tissues of vaccinated animals was significantly lower than in those of unvaccinated animals.

In the present preliminary experiments we have used techniques which attempted the quantitative assay of viable bacilli in the tissues of vaccinated and unvaccinated sheep after challenge with known numbers of *M. johnei* by the intravenous and the oral routes. As only limited numbers of sheep were available some of the experiments described here were carried out simultaneously with those reported in a previous paper (Brotherston, Gilmour and Samuel, 1961). Where appropriate, sheep used at that time to investigate the effect of dose and route of administration of *M. johnei* have been included here as unvaccinated control sheep.

MATERIALS AND METHODS

The experimental animals, strains of *M. johnei*, culture media, the preparation of infecting inocula and the viable counting technique were as described in a previous communication (Brotherston *et al.* 1961).

Vaccines. Where comparisons were made of the effects of antigens of *M. johnei* on the course of infection, animals were vaccinated once subcutaneously into the area of the brisket with 0·5 ml. heat-killed *M. johnei* in adjuvant equivalent to 3 to 4 mg. wet weight of bacilli. Vaccine was prepared by emulsifying 1 part of an appropriate saline suspension of *M. johnei* steamed for 30 minutes, with 4 parts of mineral oil (20 per cent Falba in Bayol F) which contained 0·3 per cent of phenol. In Experiment 3, a

vaccine prepared by the Central Veterinary Laboratory, Weybridge, was used in a volume of 0·5 ml., after steaming at 100°C. for 30 minutes. Oral vaccines were heat-killed cultures of the same strains of *M. johnei* used for challenge and were given as a single dose of 6 ml. equivalent to Brown's scale 20. Subcutaneous adjuvant vaccines produced a strong local reaction and tuberculin-type sensitivity which was maintained for the duration of the experiments. Oral vaccines did not stimulate skin sensitivity. Where possible, animals were vaccinated 4 to 6 weeks before challenge to permit immunity to be developed. In experiments with 3-week old lambs, vaccination was carried out just prior to challenge.

RESULTS

Effect of Vaccination on the Viable Unit Counts of Sheep after Intravenous Challenge (Exp. 1)

In this experiment 48 lambs with their ewes were run together. The untreated control lambs and challenged control lambs were those described in Experiment 1 of the previous paper (Brotherston *et al.*, 1961). The vaccinated group contained 16 lambs vaccinated at 4 weeks old and challenged at 10 weeks old, i.e. 6 weeks after vaccination. The challenged sheep were injected intravenously with 2 ml. of a 10-fold dilution of a standard suspension of *M. johnei* strain VB/4, which contained about 10^7 viable units of bacilli. Lambs from each group were killed and examined at intervals of 3 to 4, 6 to 7, 8 to 9 and 21 to 22 months after challenge. At each of these times viable unit counts of *M. johnei* were made in the ileo-caecal, the posterior mesenteric and the portal lymph nodes.

There was no evidence of infection in any of the undosed control sheep examined at the same times as the animals injected with *M. johnei*. The viable unit counts in the tissues, expressed as $\log_{10}$ units per whole node are shown in Table 1. At 3 to 4 months 2 unvaccinated animals (3548 and 3542) showed heavy colonisation of the portal and both mesenteric nodes so that the viable counts could only be estimated to be over 5 units. In the vaccinated animals, no colonies were obtained from 1 lamb (3473) and in the other (3564), 1·8 units were recovered from the portal node only. When examined between 6 to 7 months, all 4 animals in the unvaccinated group showed persistence of *M. johnei*, but there was a marked variation in the sites at which the bacilli could be detected. For example, in sheep 3543 the portal node was heavily infected, but the other sites were free or infected below the detectable level. In the vaccinated group, 2 animals (3486 and 3497) were apparently free of *M. johnei* and the other 2 showed persistence of bacilli at all 3 sites. The viable counts were not markedly less than those in some individuals of the unvaccinated group. Four animals in each group were examined between 8 to 9 months. Of those unvaccinated, only 1 (3557) was apparently free in all sites, while the others had detectable bacilli in one or other of the tissues examined. On the other hand, only 1 (3481) of 4 vaccinated animals yielded *M. johnei* on this occasion.

TABLE 1

LOG$_{10}$ VIABLE UNITS OF *M. JOHNEI* PER WHOLE NODE AFTER INTRAVENOUS INFECTION

Time in months	*Unvaccinated*				*Vaccinated*			
	No.	*Portal node*	*Posterior mesenteric node*	*Ileo-caecal node*	*No.*	*Portal node*	*Posterior mesenteric node*	*Ileo-caecal node*
3/4	3548	>5·0	>5·0	>5·0	3564	1·8	—	—
	3542	>5·0	>5·0	>5·0	3473	—	—	—
6/7	3543	5·4	—	—	3487	2·3	3·5	3·9
	3441	—	3·1	3·3	3482	1·7	3·3	2·1
	3554	4·1	2·3	4·6	3486	—	—	—
	3555	1·7	3·5	—	3497	—	—	—
8/9	3557	—	—	—	3561	—	—	—
	3549	—	3·8	3·1	3568	—	—	—
	3553	2·0	—	—	3581	1·8	3·1	3·2
	3551	2·0	3·4	3·4	3483	—	—	—
21/22	3540	—	—	—	3484	—	—	—
	3545	—	3·5	—	3491	—	—	—
	3546	—	3·0	2·1	3493	—	—	—
	3544	—	—	2·7	3571	—	—	—
	3550	—	—	1·8	3496	—	—	—
	3552	—	—	—	3558	2·0	4·6	4·8

— = less than 1·7 log$_{10}$ units.

Untreated control animals had no detectable infection at any time.

Between 21 to 22 months after challenge, the remaining animals, 6 in each group, were examined. In all of the unvaccinated group, *M. johnei* was no longer detectable in the portal nodes, but in 4 out of the 6, persistence of *M. johnei* in one or other of the intestinal nodes was demonstrated. *M. johnei* was recovered from only 1 animal (3558) of the vaccinated group.

Occurrence in the Lymph Nodes and Tissues of Vaccinated and Unvaccinated Sheep after Oral Challenge (Exp. 2)

A group of 20 lambs about 7 to 10 days old, were dosed once weekly over a period of 8 weeks with a suspension containing about 10^8 viable units of bacilli. Ten lambs of this group were vaccinated 6 weeks after the first dose to ascertain if this had any measurable effect on the course of infection. Four untreated control lambs were allowed to run with this group 1 week after the completion of dosing. Pairs of lambs from the vaccinated and unvaccinated groups were killed and examined at intervals between 10 weeks and 53 weeks after the first dose was given. An untreated animal was also examined at each period. On each occasion, the viable counts were calculated per gramme of (a) the posterior mesenteric group of nodes; (b) the mesenteric nodes; (c) the portal node; (d) the liver and (e) the spleen.

TABLE 2

M. JOHNEI IN LYMPH NODES OR TISSUES OF UNVACCINATED AND VACCINATED LAMBS

Dosed orally with 10^8 viable units per week for eight weeks

Weeks after first dose	*Unvaccinated*				*Vaccinated*			
	Log_{10} viable units per g.				*Log_{10} viable units per g.*			
	No.	*Posterior mesenteric node*	*Mesenteric node*	*Portal Spleen Liver*	*No.*	*Posterior mesenteric node*	*Mesenteric node*	*Portal Liver Spleen*
10	152	3·2	2·6	—	4957	3·3	3·7	—
10	4959	3·6	3·8	—	4958	2·9	3·9	—
16	4990	5·2	3·6	portal 2·6	4975	3·2	2·6	—
16	4977	—	2·6	—	4965	2·6	2·6	—
24	4972	—	2·6	—	4952	—	—	—
24	4979	—	2·6	—	4986	—	2·6	—
32	4964	—	2·6	—	4951	—	2·6	—
32	4980	3·0	3·6	—	4976	—	—	—
53	4962†	5·3	5·1	—	4966	—	—	—
53	4987	Died other causes			4989	—	2·6	—

One untreated control animal was killed at each time starting at 16 weeks. One showed detectable infection in the mesenteric nodes at 24 weeks (2·6 viable units per g.); the others remained free.

† Clinical case.

— Less than $\log_{10}$ 1·7 viable units.

The results of examinations of vaccinated and unvaccinated animals are shown in Table 2. The first 4 animals (2 vaccinated and 2 unvaccinated) were about 10 weeks old and all 4 had similar viable counts in the lymph nodes of the intestine. In subsequent examinations at 16, 24 and 32 weeks there was no observable difference between the viable unit counts per g. of those vaccinated and non-vaccinated animals in which *M. johnei* was detectable. The exception to this was 1 unvaccinated sheep (4962) which was a clinical case and had over 5 $\log_{10}$ viable units per g. in both lymph nodes. *M. johnei* was not recovered from the liver or spleen of any animal. Of the control sheep, 1 of which was examined at each time interval, 1 only showed evidence of infection at 24 weeks when there was a viable count of 2·6 units per g. in the mesenteric nodes.

Effect of Vaccination on the Viable Unit Counts in Lambs dosed Orally at 3 Weeks and 3 Months (Exp. 3)

This experiment was carried out in 2 parts. In the first, lambs were given vaccine at about 2 weeks old and at 3 weeks old they were dosed orally each week for 10 weeks. In the second, lambs were given vaccine at 2 months old, and at 3 months old they were dosed orally each week for 10 weeks. The experimental groups were as follows:

Three-week old lambs: Group 2, dosed only with *M. johnei*; Group 2a, given oral vaccine and dosed; Group 2b, given subcutaneous adjuvant vaccine and dosed.

Three-month old lambs: Group 4, dosed only with *M. johnei*; Group 4a, given oral vaccine and dosed 1 month later; Group 4b, given subcutaneous adjuvant vaccine and dosed 1 month later.

The standard suspensions were prepared from *M. johnei* strain 101/2 for the 3-week old lambs and 101/3 for the 3-month old lambs. In both age groups about 10^5 viable units were given once weekly for 10 weeks. Two untreated control lambs were placed in contact with Group 2 for the duration of the dosing period and until they were killed at the first examination at 1 month after the last dose. Three additional control lambs were then introduced into Group 2, 1 of which was examined at 4 to 5 months and 2 at 8 to 9 months after the last dose. As no contact control sheep showed infection, controls were not included in the experiment with Group 4 which was started after the first experiment had been completed. Four or 5 animals from each group were killed and examined at 1 month, 4 to 5 months and 8 to 9 months after the last infective dose. The viable unit counts per g. of the mesenteric nodes and of the whole small intestinal mucous membrane were made as described by Brotherston *et al.* (1961). The retropharyngeal nodes were examined for the presence or absence of *M. johnei*.

The effects of vaccination on the viable unit counts in the small intestine and the associated lymph nodes of lambs first dosed at 3 weeks of age are shown in Table 3. The viable counts are expressed as the $\log_{10}$ units of *M. johnei*. In the intestine, the minimum detectable number of units was 4·18 because the lowest dilution used was 10^{-1} of the homogenate. When examined 1 month after the last dose, the lambs given oral vaccine had viable counts in the mesenteric lymph nodes which varied from no detectable units in 2 individuals (6176 and 6174) to 3·55 units per g. All 4 animals showed infection of the intestine which varied from 4·78 to 6·26 units. In the lambs given subcutaneous adjuvant vaccine, the counts in the lymph nodes varied from no detectable units in 1 animal (6189) to 2·09 units per g. In the intestine the counts varied from no detectable units in 2 animals (6187 and 6190) to 4·48 units. Unvaccinated lambs had counts in the lymph nodes which varied from no detectable units in 2 animals (6161 and 6163) to 3·74 units per g. In the intestine all 4 animals were shown to be infected, the counts varying from 5·35 to

TABLE 3

EFFECT OF VACCINATION ON THE VIABLE UNIT COUNTS OF *M. JOHNEI*

Small intestine and the associated lymph nodes of lambs vaccinated and dosed at three weeks of age

Time after last dose	*Group 2a: oral vaccine*			*Group 2b: s/c adjuvant vaccine*			*Group 2: unvaccinated controls*		
	No.	*Nodes per g.**	*Small intestine**	*No.*	*Nodes per g.*	*Small intestine*	*No.*	*Nodes per g.*	*Small intestine*
1 month	6176	—	4·78	6187	1·7	—	6160	2·69	5·35
	6174	—	5·73	6190	2·0	—	6162	3·74	5·67
	6175	3·55	6·01	6188	2·09	4·18	6161	—	5·82
	6177	2·0	6·26	6189	—	4·48	6163	—	6·63
4–5 months	6180	1·7	—	6192	—	—	6166	—	—
	6181	—	4·18	6194	—	—	6169	—	—
	6178	—	4·18	6195	—	—	6170	—	—
	6182	2·0	4·18	6196	—	—	6167	1·7	4·18
	6179	3·74	4·18	6191	—	—			
8–9 months	6184	—	—	6193	—	—	6164	—	—
	6172	—	—	6197	—	—	6165	—	—
	6186	1·7	—	6199	—	—	6168	—	—
	6185	2·60	—	6198	—	—	6171	—	4·65
	6183	—	4·65	6200	2·30	6·21			

* Log_{10} units per g. of lymph node and length of small intestine minimum detectable units 1·7 and 4·18 respectively.

Uninoculated control animals were included in Group 2 at each period. None became infected.

6·63 units. In respect of the comparison between viable unit counts of the lymph nodes, no difference was detectable at 1 month after the last dose as a result of the treatment given to the groups of lambs. In the small intestine of animals in Group 2b, given subcutaneous adjuvant vaccine, at 1 month the detectable viable units were much less than in either of the other 2 groups. At 4 to 5 months 1 animal in Group 2, no animals in Group 2b and all animals in Group 2a were infected. At 8 to 9 months, 1 animal in Group 2, 1 animal in Group 2b and 3 animals in Group 2a were infected. The retropharyngeal lymph nodes were examined from each animal at each period, but *M. johnei* was not detected. The untreated control sheep had no detectable infection when examined at the same times as the other animals.

The effects of vaccination on the viable unit counts lambs in first dosed at 3 months of age are shown in Table 4. Exactly the same conditions and methods were applied to these animals as has been described in the above experiment, except that dosing was delayed for 1 month after vaccination. At 1 month after dosing the Group 4b which had received treatment with subcutaneous adjuvant vaccine showed a decided advantage over the other 2 groups. In only 2 animals (6149 and 6288) was infection detectable and it was

TABLE 4

EFFECT OF VACCINATION ON THE VIABLE UNIT COUNTS OF *M. JOHNEI*

Small intestine and the associated lymph nodes of lambs vaccinated at two months and dosed at three months of age

Time after last dose	*Group 4a: oral vaccine*			*Group 4b: s/c adjuvant vaccine*			*Group 4: unvaccinated controls*		
	No.	*Nodes per g.**	*Small intestine**	*No.*	*Nodes per g.*	*Small intestine*	*No.*	*Nodes per g.*	*Small intestine*
1 month	6154	2·69	—	6151	—	—	6148	4·11	5·69
	6253	2·65	5·18	6144	—	—	6248	3·60	5·96
	6250	—	5·61	6147	—	—	6267	2·60	6·33
	6155	3·19	5·72	6149	1·7	—	6247	3·28	6·57
	6145	—	6·29	6288	3·0	—			
4–5 months	6283	—	—	6254	—	—	6262	—	—
	6277	—	—	6251	—	—	6252	—	—
	6143	—	—	6266	—	—	6261	2·0	—
	6247	—	—	6286	—	4·78	6263	—	—
	6275	—	4·18	6259	—	5·48			
8–9 months	6255	1·7	4·78	6268	—	4·47	6276	—	—
	6257	—	—	6272	—	—	6279	—	—
	6260	2·09	4·96	6278	—	—	6280	2·0	4·27
	6264	—	—	6281	—	—	6282	—	—
	6270	—	—	6287	—	—			

* Log_{10} units per g. of lymph node and length of small intestine minimum detectable units 1·7 and 4·18 respectively.

TABLE 5

DISTRIBUTION OF INFECTION

Mesenteric lymph nodes and small intestine of sheep found infected after oral dosing

	Nodes + Intestine +		*Nodes − Intestine +*		*Nodes + Intestine −*	
All orally dosed sheep ..	28/60	46%	21/60	35%	11/60	18%
Unvaccinated sheep ..	18/28	64%	7/28	25%	3/28	10%

Numerator = number infected in each category.

Denominator = total number examined and found infected.

confined to the mesenteric lymph nodes. In Groups 4a and 4, the orally vaccinated and the unvaccinated groups, all the animals showed detectable viable unit counts in the mesenteric lymph nodes and the intestine. The level of infection was comparable to that described in the younger animals (Table 3), varying from no detectable units to 4·1 units per g. of lymph node and from 5·18 to 6·57 units in the intestinal mucous membrane. The one exception was lamb 6154 of Group 4a, in which no infection was detectable in

the intestine. At 4 to 5 months after dosing 1 animal in Group 4, 2 animals in Group 4b and 1 in Group 4a were infected. At 8 to 9 months 1 animal in Group 4, 1 in Group 4b and 2 in Group 4a were infected. The presence of *M. johnei* was not detectable in the retropharyngeal nodes of any animal.

DISCUSSION

The purpose of these experiments was to compare the viable unit counts of *M. johnei* in the tissues of unvaccinated and vaccinated sheep at various times after intravenous or oral infection. It was considered inadvisable to use live antigen as vaccine because of the possibility of dissemination of the antigen from the site of vaccination.

In Experiment 1 no clinical case of Johne's disease occurred, so that the challenge dose of about 10^7 viable units of bacilli was unlikely to have overwhelmed any low grade immunity resulting from vaccination. At 3 to 4 months after intravenous challenge very large numbers of viable units were recovered from the portal and from the mesenteric lymph nodes of the 2 unvaccinated lambs, whereas in the 2 vaccinated animals only a few colonies were recovered from the portal lymph node of 1 lamb. However, at periods later than 3 to 4 months, *M. johnei* was being slowly eliminated from the tissues of vaccinated and unvaccinated sheep. This suggests that the main effect of vaccination was to promote the earlier clearance of *M. johnei* from the sites to which they had been carried by the blood, whereas in unvaccinated animals *M. johnei* was cleared at a slower rate. It is unfortunate that only 2 animals in each group were examined at 3 to 4 months but differences between groups were anticipated at later periods when larger numbers of sheep were examined.

It is interesting to compare the incidence of detectable infection in individuals of the vaccinated and unvaccinated groups examined at different times after challenge. In none of the individual groups killed at 3 to 4 months, 6 to 7 months, 8 to 9 months or 21 to 22 months after infection were the differences significant, but over the whole experiment the cumulative totals showed that of 16 unvaccinated sheep, *M. johnei* was detected in 13 compared with 5 in the vaccinated group ($X^2=8{\cdot}13$ $P<0{\cdot}01$).

In Experiment 2 groups of vaccinated and unvaccinated lambs were given repeated oral doses to simulate conditions approaching those to which lambs might be exposed in an infected environment. For this reason too vaccination took place during the process of challenge. There were no differences in the viable counts in unvaccinated and vaccinated lambs examined up to 32 weeks after dosing. By 53 weeks the 1 surviving animal in the unvaccinated group had become a clinical case, with over 5 $\log_{10}$ viable units per g. in both lymph nodes. On the basis of a quantitative examination of the mesenteric lymph nodes, however, vaccination was ineffective.

In Experiments 3 and 4 the viable unit counts in the intestinal

mucous membranes, in addition to the mesenteric lymph nodes of 3-week old and 3-month old lambs given oral and subcutaneous vaccines, were compared with those of untreated animals. Experiment 3 was run concurrently with those which described the effects of different levels of oral infection (Brotherston *et al.* 1961). At 1 month after dosing the lambs in the group vaccinated subcutaneously were apparently able to limit the multiplication in the intestine so that no viable bacilli were detectable in 2 lambs and in the other 2 the viable counts were the minimum detectable by the technique. In both the orally vaccinated and in the untreated lambs the intestinal counts were much higher. Vaccination with adjuvant vaccine was not able to prevent the invasion of the mesenteric lymph nodes which followed oral dosing. A comparison of the viable unit counts in lambs examined at 4 to 5 and 8 to 9 months after dosing could not be made because bacilli were detectable in only a few animals in each of the vaccinated and unvaccinated groups. In a previous communication (Brotherston *et al.* 1961) it has been pointed out that an oral challenge dose of 10^6 viable units can initiate an infection which is observable 1 month later, but is unlikely to produce progressive infection in the majority of animals at later periods.

In analysing the results of the comparative viable counts in vaccinated and unvaccinated lambs first dosed at 3 months of age the same conditions and the methods of the preceding experiment were applied except that the animals were vaccinated 1 month before challenge commenced. It is encouraging, therefore, that this experiment which was carried out after that described in lambs, confirmed the previous result that subcutaneous adjuvant vaccine effectively limited the establishment of *M. johnei* in the intestine. Again the effect could be demonstrated only at 1 month after the last challenge dose. The results from 3-month old lambs also confirmed that vaccination did not prevent the invasion of the mesenteric lymph nodes which followed oral dosing and that comparisons which are confined to an examination of these nodes are not suitable for the evaluation of vaccines. Since 3-month old lambs appeared to be as susceptible as 3-week old lambs to oral dosing there would seem to be no age immunity to oral infection at least up to 3 months of age.

Oral vaccines in the form used were not shown to be effective in either experiment; indeed at 4 to 5 months, there was some evidence of an increased susceptibility in sheep so treated, but since the minimum detectable viable count in the intestinal mucosa was $\log_{10}$ 4·18, this could be a matter of chance in this particular experiment.

At this point it is possible to discuss more fully the distribution of infection in the mesenteric lymph nodes and the intestines of sheep described in the paper by Brotherston *et al.* (1961), (Experiment 3, Table 4), and in the present paper on vaccination. The distribution of infection in all lambs dosed orally and the distribution of infection when confined to unvaccinated lambs are shown in Table 5.

Forty-six per cent of all orally dosed sheep and 64 per cent of unvaccinated sheep were infected in both the intestine and the associated lymph nodes. In 35 and 25 per cent respectively, infection was confined to the intestine and in 18 and 10 per cent infection was detectable only in the lymph nodes. Evidence of infection based on an examination of mesenteric lymph nodes alone would have missed 20 to 30 per cent of infected animals. Therefore the intestinal mucous membrane of sheep must be examined in such experiments. This distribution supports evidence that the intestinal mucous membrane is the predilection site of multiplication of *M. johnei* in sheep, at least in the primary preclinical stages of the disease. In view of this evidence perhaps there should be a reassessment of the occurrence of *M. johnei* in apparently normal cattle, particularly in calves, in which the infection rate is based upon the culture of viable bacilli from the mesenteric lymph nodes (Cunningham and Gilmour, 1959, and others).

Investigations into immunity and resistance of animals to infections with Mycobacterium species have often given conflicting results. According to Dubos *et al.* (1953) none of the methods of anti-tuberculosis vaccination is sufficiently effective to protect all animals completely against experimental infections, but low levels of immunity can be measured by following quantitatively the fate of bacilli in the tissues of vaccinated and non-vaccinated animals. This is true also of immunity in relation to infection with *M. johnei* and it is evident that there are many variables which have an important bearing upon the observable effects of vaccination on the preclinical stages of Johne's disease. For example, the results suggest that the immunity stimulated is of low grade and the probable site of primary infection with *M. johnei* is the intestine. Clearly then, the size of the challenge dose and the sites at which to observe the effects of vaccination are two very important factors in experimental infections of sheep. Even when the challenge dose is small a few vaccinated animals are unable to destroy completely the bacilli which survive in the tissues when these are examined some months after the challenge dose. This is probably the result of variation to the stimulus provoked by *M. johnei* in individual animals. Observations made during the preclinical stages and preferably within 2 months of the challenge, give optimum results. It is encouraging that vaccination can be shown to limit multiplication of *M. johnei* in the early stages of primary infection even when only a low grade of immunity is induced by vaccination, and even though this can only be measured between 1 and 4 months after challenge.

CONCLUSIONS

By quantitative assay of *M. johnei* in the mesenteric and portal lymph nodes it was possible to show an increased clearance of *M. johnei* from the tissues of sheep challenged intravenously, but no such effect was shown in the orally challenged sheep. *M. johnei* could not

be detected in the liver or spleen of sheep given *M. johnei* by the oral route.

In an experiment involving the examination of the intestinal mucosa and mesenteric lymph nodes of sheep infected orally at 3 weeks and 3 months of age, subcutaneous adjuvant vaccine enhanced the sheep's ability to overcome intestinal infection while oral vaccination did not. Only by comparing viable unit counts from the intestinal mucosa at 1 month after the last challenge dose could the effect of vaccination be demonstrated. Vaccination did not prevent the invasion of the lymph nodes of sheep challenged orally with *M. johnei*. The distribution of *M. johnei* in the mesenteric lymph nodes and intestinal mucosa was such that examination of the nodes alone would have missed a number of infected sheep. Lambs dosed at 3 months of age were as susceptible to oral infection as those dosed at 3 weeks of age.

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AN IMMUNOLOGICAL PHENOMENON IN CHICKENS FOLLOWING INFECTION WITH *MYCOPLASMA GALLISEPTICUM*

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INTRODUCTION

Immunity against infection with *Mycoplasma gallisepticum* was probably first demonstrated by Nelson (1935). Although at that time unaware of the aetiology, he observed that chickens which had recovered from coryza induced by intranasal exposure to exudate harvested from diseased chickens were resistant to re-exposure. In Australia, Hart (1940) was unable to produce disease in the sinuses of two turkeys which had recovered from experimental infection in those sinuses. Such immunity was not stimulated either by the subcutaneous inoculation of exudate from diseased birds, or by its application with friction to the cloacal mucous membrane. Significant immunity to reinfection was reported by Delaplane and Stuart (1943) in chickens which had recovered from chronic respiratory disease (CRD)†. Fahey and Crawley (1954), using a strain of *Mycoplasma* and a virus in consort, did not observe symptoms in chickens challenged after recovery from a previous exposure. An identical challenge in susceptible chicks produced a mortality of 60 per cent. (This "disease" is, of course, far removed from the relatively mild coryza originally described by Nelson (1935)). The same workers subsequently suggested that the presence of persistent "carriers" of *Mycoplasma* indicated that no worthwhile immunity was produced under natural conditions (Crawley and Fahey, 1957).

However, immunity in chickens following recovery from natural infection has been reported (Van Roekel and Olesiuk, 1952), although this may not be of a high order (Olesiuk, Van Roekel and Gray, 1953), and is slow to develop (Tudor, 1953). At this time, it was suggested that control of the disease by means of immunisation might be a possibility (Van Roekel and Olesiuk, 1953).

Prier and Dart (1951) attempted to immunise turkeys against sinusitis by using exudate or macerated infected embryos; the results were inconclusive. Domermuth (1957) inoculated chicks intramuscularly or subcutaneously with *Mycoplasma* propagated either in embryonating chicken eggs or in artificial media. In spite of his suggestion that the subsequent challenge into the posterior thoracic air-sac was too severe, a definite immune response was observed. Adler (1958) inoculated turkeys intramuscularly with a culture of *M. gallisepticum* inactivated with 0·25 per cent. formalin, either alone, or with various adjuvants. Little or no serological response was detected by the agglutination test and all the

*This work formed part of a thesis for the PH.D. degree of the University of California.

†It is probable that the CRD now attributed to *M. gallisepticum* is not the same disease originally described by Delaplane and Stuart. Delaplane's later comment (1952), that there was no evidence of substantial immunity in CRD, almost certainly applied to *Mycoplasma* infection. In this paper, CRD is used in that sense.

birds were susceptible to intraperitoneal challenge. A second attempt with viable organisms, attenuated by ninety passages in artificial media and inoculated intramuscularly, likewise evoked only a poor agglutinin response and no demonstrable immunity. Adler, McMartin and Shifrine (1960) concluded that inactivated organisms, when inoculated intravenously, intramuscularly, or into the Bursa of Fabricius, would not immunise chickens, nor protect turkeys when inoculated intramuscularly. Whole culture of the organism, attenuated by about fifty passages in artificial media and inoculated intramuscularly, failed to protect turkeys against challenge in the abdominal air-sac six weeks later, although chickens similarly inoculated were resistant for approximately two months. It was further shown that chickens, inoculated in the abdominal air-sac with *M. gallisepticum* four months previously, maintained their pre-inoculation level of egg production on challenge, while that of susceptible controls dropped by about 12 per cent.

It was stated recently that immunisation had given very little promise of controlling or preventing CRD (Olesiuk and Van Roekel, 1960). This may be true, but one can scarcely say that the subject has been adequately investigated. The following experiments were designed to evaluate in a quantitative manner immunity of the air-sacs of chickens, following controlled infection with *M. gallisepticum*.

MATERIALS AND METHODS

Chickens. Single Comb White Leghorn chicks were obtained at one day of age from the Department of Poultry Husbandry in Berkeley. They were held in isolation facilities at Davis until required for experimental purposes at four weeks of age. The chickens were then wing-banded and blood samples taken from the wing vein to test for serum agglutinins for *M. gallisepticum*. Groups were housed on wood shavings, with a constant air temperature of 70°F. Feed and water were provided *ad lib.*

Culture media. The basal ingredients in liquid and solid media were respectively, PPLO broth and PPLO agar (Difco), enriched with 1 per cent. yeast autolysate (Albimi) and 10 per cent. horse serum. The liquid medium contained in addition 0·025 per cent. dextrose. The pH was adjusted to 7·6.

The medium used to find the concentration of organisms in a culture differed from the preceding in that the concentration of dextrose was 0·5 per cent. and contained, in addition, phenol red indicator, thallium acetate to a concentration of 1:4,000, and 500 units of sodium penicillin per ml. One ml. of culture was diluted serially through 10 tubes containing 9 ml. of medium. From the dilutions, 0·02 ml. was cultured on solid medium, because occasionally the liquid medium failed to support growth. About seven days elapsed before fermentation was complete and the concentration of organisms known.

For re-isolation of *M. gallisepticum* from body tissues, cultures were made in tubes containing 3 ml. of a medium identical to that just described, except that the concentration of dextrose was 0·025 per cent. Subsequent treatment of the culture was in accordance with the procedure of Adler, Yamamoto and Bankowski (1954). To ensure that the strain of *Mycoplasma* isolated was the same as that inoculated, broth cultures were propagated periodically from single colonies picked from agar plates. From these, agglutinating antigens were prepared and tested against

known positive and negative sera. Dilutions of culture for inoculation were made in sterile tryptose broth.

Source of organism. The S6 strain of *M. gallisepticum* was used in all experiments. A series of cultures was propagated from a single colony picked from a serum agar plate, following initial isolation in broth from experimentally induced air-sac lesions in chickens. Purity of the isolate was tested culturally, biochemically and serologically; its pathogenicity for chickens was affirmed. A stock was then lyophilised at the 4th in vitro passage in non-inhibitory medium. Following reconstitution, no culture was passaged more than six times before being inoculated into chickens.

Inoculation of chickens. Immunisation was performed by intranasal instillation of culture, suitably diluted. To expose a large area of the mucosa, 0·25 ml. was deposited in each nostril and 0·5 ml. injected with moderate force into the palatine cleft, giving a total volume of 1 ml. Challenge was directly into the left abdominal air-sac, and also comprised suitable dilutions of culture in doses of 1 ml.

Serology. An agglutinating antigen was prepared from the 3rd in vitro passage of the series described above, using the method of Adler (1958), except that 1:10,000 merthiolate was added as the preservative instead of 0·25 per cent. phenol. Following the addition of crystal violet, the antigen was left at room temperature overnight. It was then centrifuged at 1,000 r.p.m. for five minutes which removed extraneous particulate material. Standardization was carried out against doubling dilutions of known positive and negative sera. The antigen was stored in volumes of 4 ml. at 4°C. Before use, the contents of each vial were tested, as before, against the same sera, aliquots of which were held at —40°C. Over a period of nine months, there was no alteration in the specificity or sensitivity of this antigen.

Slide agglutination tests were performed using the method of Adler (1954). Titres were obtained by making doubling dilutions of serum in physiological saline, commencing with 1:10. Tests were conducted on certain samples of serum for neutralizing antibodies against the viruses of Newcastle disease (ND) and infectious bronchitis (IB). In no case was a diagnostic level found.

RESULTS

Active Immunisation of Air-Sacs: Measurement of Minimum Immunising Dose (Exp. I)

The objectives were to determine whether intranasal inoculation of *M. gallisepticum* would immunise chickens against homologous challenge in the abdominal air-sacs and, if so, to measure approximately how many organisms were necessary to produce such resistance.

The first requirement was an estimate of a reasonable challenge dose. It was decided that this should be the number of organisms which would produce visible lesions in the air-sacs of 50 per cent. of chickens, when the inoculum was introduced directly into the abdominal air-sac (AID_{50}). To measure this, 7 groups of 6 chickens each, four weeks of age, were inoculated in the left abdominal air-sac with 1 ml. of logarithmic dilutions of the 6th in vitro passage of *M. gallisepticum,* of which the undiluted culture had a concentration

TABLE 1

EXPERIMENTAL RESULTS IN THE CALCULATION OF AN AID^{50}.*

Group No.	*No. of organisms inoculated*	*Bird number*	*Serum agglutinin titre*	*Presence of active lesions*	*Isolation of organism*
1	10^7	1	80	+	+
		2	80	+	+
		3	80	+	+
		4	80	+	+
		5	40	+	+
		6	20	+	+
2	10^6	1	160	+	+
		2	40	+	+
		3	40	+	+
		4	40	+	+
		5	40	+	+
		6	40	+	+
3	10^5	1	80	+	+
		2	80	+	+
		3	80	+	+
		4	80	+	+
		5	40	+	+
		6	20	+	+
4	10^4	1	20	+	+
		2	20	+	+
		3	20	+	+
		4	20	+	+
		5	10	+	+
		6	+	+	+
5	10^3	1	40	+	+
		2	40	+	+
		3	40	+	+
		4	20	+	+
		5	20	+	+
		6	20	+	+
6	10^2	1	20	+	+
		2	10	+	+
		3	+	—	—
		4	+	—	—
		5	+	—	—
		6	+	—	—
7	10^1	1	10	+	+
		2	+	+	+
		3	+	+	+
		4	—	—	—
		5	—	—	—
		6	—	—	—

*In this instance, $AID_{50} = 10^{1.8}$ organisms.

of approximately 10^8 organisms per ml. Blood samples were taken after ten days and the sera tested for agglutinins. Four days later, further samples from those chickens which had been negative on the

first test, were again negative. Accordingly, autopsy was performed; the presence or absence of air-sac lesions was recorded; cultures were made from the left abdominal air-sac of every bird; certain representative tissues were placed in 10 per cent formalin for fixation, sectioning and staining with haematoxylin and eosin. The principal results are shown in Table 1. By the method of Reed and Muench (1938), the AID_{50} was calculated to be approximately $10^{1.8}$ organisms. It should be pointed out that while an active air-sac lesion was invariably accompanied by a serological response, the converse did not always hold, although these exceptions were confined to the higher dilutions. Histological examination revealed no definite evidence of disease in these cases. *M. gallisepticum* was isolated in every instance from active lesions but not from grossly normal air-sacs. Agglutination titres are included for comparison of individuals within groups. Obviously, titres between groups cannot be compared because of the range in concentration of the inocula. However, active air-sac lesions produced by 10^2 organisms were no less severe than those resulting from 10^7 (Fig. 1).

Intranasal inoculation was carried out with the same dilutions of culture as were employed in measurement of the AID_{50}, using 8 groups (Nos. 1 to 8) of 10 chickens from the same hatch as that utilized above. An additional group (No. 9) of 8 chickens was exposed to undiluted culture, to gauge the severity of disease which might at most be obtained by this route of inoculation. To obtain evidence of bird-to-bird transmission within groups, one uninoculated chicken was placed in contact with each of groups 1 to 6, and 9, on the day following inoculation. These birds were observed as groups for signs of respiratory disease every two days; on the 10th day blood samples were taken and on the 17th day every individual was examined for evidence of coryza.

The culture which had been used in the initial inoculations was now at the 10th in vitro passage. Using the calculated AID_{50} as a gauge, dilutions were made from 10^{-5} to 10^{-8}. Two sub-groups were formed from each group and these were then challenged in the left abdominal air-sac, fifteen days after intranasal exposure, with 1 ml. of either 10^{-5} or 10^{-6} dilution. Group 9 was not challenged. As challenge controls, 2 groups of 10 birds and 2 groups of 5 birds, again from the same hatch, were inoculated similarly with dilutions 10^{-5} to 10^{-8} respectively. The approximate concentration of organisms in each dilution was calculated as before. Seven days after challenge, a blood sample was taken from the wing vein of every bird to test for the presence, and titre, of serum agglutinins. Excess mucous in the nasal cavity was revealed by slight pressure over the turbinate bones. Autopsy was then performed special attention being paid to the respiratory tract. Cultures were made from (i) the left abdominal air-sac and (ii) the nasal cavity and infraorbital sinuses; representative portions of tissue from the same areas were placed in 10 per cent formalin for fixation, sectioning and staining with haematoxylin and

TABLE 2

RESISTANCE TO CHALLENGE OF CHICKENS IMMUNISED WITH VARYING NUMBERS OF ORGANISMS

Group No.	*Approximate number of organisms used to immunise*	*Pre-challenge agglutination response*	*Active air-sac lesions following challenge with varying doses*				*Isolation of organism from air-sacs*	*Nasal passages*		
			No. of organisms					*Coryza*	*Isolation of organism*	*Group histopathology*
			10^4	10^3	10^2	10^1				
1	10^7	9/9*, **	0/5	0/4			0/9	2/9	1/9	+
2	10^6	10/10	0/5	0/5			0/10	0/10	0/10	+
3	10^5	10/10	0/5	0/5			0/10	0/10	3/10	+
4	10^4	7/10	0/5	0/5			0/10	0/10	3/10	+
5	10^3	5/10	1/5	0/5			1/10	0/10	2/10	+
6	10^2	5/10	2/5	2/5			4/10	0/10	0/10	+
7	10^1	2/10	3/5	5/5			7/10	0/10	0/10	+
8	10^0	0/10	3/5	4/5			7/10	0/10	0/10	—
9	Susceptible Controls	0/30	7/10	6/9**	1/5	0/5	13/29	0/29	0/29	—

*Numerator—number positive.
Denominator—number tested.

**One bird in this group died for undetermined reason.

eosin. In addition, cultures and tissues were taken from the tracheas of the chickens of Group 9.

The principal results are shown in Table 2. The challenge culture was estimated to have a concentration of 10^9 organisms per ml. From this figure and the level of air-sac lesions produced in the control birds, and assuming that all lower dilutions would have produced lesions in every bird, the AID_{50} was calculated to be approximately $10^{2.9}$ organisms. This result differs by only one logarithm from that obtained in the initial calculation of the AID_{50}. It follows then, that 10^{-5} and 10^{-6} dilutions contained approximately 11 and 1.1 AID_{50} per ml. respectively.

It is evident that sub-groups 1 to 5 completely resisted 1.1 AID_{50}; sub-groups 1 to 4 similarly resisted 11 AID_{50}, significant protection being present in sub-group 5. (At $p=0.05$, adjusted $X^2=3.92$, *cf.* tabular value of 3.84). Although some individuals had responded with the production of agglutinins to intranasal exposure at higher dilutions, protection against challenge was not significant. The minimum immunising dose (MID), as estimated by a challenge close to one AID_{50}, was approximately 10^3 organisms intranasally. This is markedly less than the figure of 10^7 organisms obtained by Adler *et al.* (1960) for intramuscular vaccination. Sites of local invasion were observed occasionally in the air-sacs of immune birds (Fig. 5). However, *M. gallisepticum* was not isolated from these; histological examination revealed that reaction was confined within the wall of the air-sac and exudation was virtually absent. Almost every active air-sac lesion yielded *M. gallisepticum* on culture.

Coryza was observed only in a few birds of Group 1, but organisms were isolated from the nasal passages of some birds up to Groud 5. As indicated by a serological response in the uninoculated chickens of groups 1, 2, 4 and 5, bird to bird transmission probably occurred at least up to group 5. This may be of importance in the case of some chickens which, serologically negative at ten days, became positive after challenge, but showed no active air-sac lesions at autopsy. However, it cannot be certain that this resistance is a result of contact infection since the greatest challenge dose employed was not sufficient to produce lesions, or even a serological response, in all control birds.

Histopathological changes in the form of glandular hypertrophy, exudation, mucosal and submucosal infiltration with mononuclear cells which showed a tendency towards lymphoid follicle formation, were found in the nasal cavity and infra-orbital sinuses of birds up to group 6. Thereafter, it became difficult to distinguish the normal from the abnormal, although strongly suggestive cellular infiltration was present in the submucosa of some birds in group 7.

Of the 8 chickens inoculated intranasally with 10^8 organisms twenty-five days previously, only one had left sided air-sac lesions at autopsy with a concurrent caseous pneumonia on the same side. Histological examination of the lung and air-sac revealed that the

disease processes had reached a granulomatous stage and were therefore not of recent origin. This point is believed to be of some importance in considering the nature of the immune response and will be raised again in the discussion. Four out of the 8 chickens developed coryza between the 17th and 25th days after inoculation. Surprisingly, the uninoculated control bird did not become infected.

Chronological Development and Effectiveness of Active Immunity (Exp. 2)

While this work was to some extent confirmation of the results obtained in Experiment 1, two further objectives were aimed at, namely to determine how soon and to what degree, immunity developed.

Four groups of 15 chickens (groups 1 to 4), and one group of 20 chickens (group 5), were inoculated intranasally with 1 ml. of a 10^{-1} dilution of the 6th in vitro passage of *M. gallisepticum*. The dose was calculated to be 10^8 organisms. On the 2nd, 5th, 7th, 11th and 21st days after exposure, blood samples were taken from the chickens in groups 1, 2, 3, 4 and 5 respectively. At these times sub-groups, usually of 5 birds each, were formed. Members of the sub-groups were challenged in the left abdominal air-sac with 1 ml. of various dilutions of culture of *M. gallisepticum* (Table 3). To estimate the severity of each challenge, groups of susceptible chickens were inoculated similarly with selected dilutions. Blood samples were taken and autopsy performed as in Experiment 1 between six and nine days after challenge of each group. The results are shown in Table 3.

There was no evidence of immunity two days post-vaccination, but by the fifth day, at which time agglutinins were detectable in the sera of some vaccinated individuals, considerable resistance had developed. This was indicated by diminution in the extent of the lesions resulting from challenge (Fig. 2). While no immunised bird resisted completely, it must be pointed out that even the smallest challenge was considerably in excess of 100 AID_{50}. Evidence of more substantial immunity was gained at seven and eleven days post-vaccination (Figs. 3 and 4). At 21 days, no bird developed active air-sac lesions following challenge with approximately 3, 30, or 300 AID_{50} (Fig. 6).

Once again, re-isolation of *M. gallisepticum* from the active lesions in the air-sacs was achieved in a high proportion of cases, including those in which some immune response had already taken place. However, in one instance only, *M. gallisepticum* was isolated in the absence of active lesions from a bird challenged seven days post-vaccination with approximately 10^6 organisms. With regard to recoveries from the nasal cavity and infraorbital sinus, poor results were obtained in the first group where heavy contamination occurred, attributed to insufficient autoclaving of the cotton swabs. During the 2nd, 3rd and 4th weeks frequent isolations were made. In only one control bird was an active air-sac lesion not accompanied

TABLE 3

DEVELOPMENT OF IMMUNITY IN THE AIR-SACS FOLLOWING INTRANASAL EXPOSURE

Group No.	*Days after exposure*	*Subgroups: V=vaccinated S=susceptible*	*Active air-sac lesions following challenge with varying doses** — *Number of organisms* 10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	*Isolation of organism from air-sac*	*Nasal passages* — *Coryza*	*Nasal passages* — *Isolation of organism*	*Remarks*
1	2	V1	5/5								5/5	0/5	1/5	Nasal cultures in whole group contaminated.
		V2			5/5						5/5	0/5	0/5	
		V3					5/5				4/5	0/5	0/5	
		S1			5/5						3/5	0/5	0/5	
		S2					5/5				3/5	0/5	0/5	
		S3							5/5		2/5	0/5	0/5	
		S4								2/5	2/5	0/5	0/5	
2	5	V1	5/5								3/5	0/5	4/5	Diminution of lesions in some immunised birds. See Fig. 2.
		V2			5/5						4/5	0/5	3/5	
		V3					5/5				5/5	0/5	5/5	
		S1							5/5		4/5	0/5	0/5	
3	7	V1		4/5							4/5	3/5	5/5	Lesions in immunised birds much less extensive than in controls. See Fig. 3.
		V2				3/5					4/5	3/5	5/5	
		V3						5/5			5/5	1/5	4/5	
		S1								5/5	4/5	0/5	0/5	
4	11	V1		5/5							5/5	0/5	5/5	Lesions in some immunised birds confined to very small area. See Fig. 4.
		V2				3/5					3/5	1/5	3/5	
		V3						2/5			2/5	3/5	5/5	
		S1								5/5	4/5	0/5	0/5	
5	21	V1					0/4				0/4	1/4	2/4	All immunised birds resisted maximum challenge of approximately 330 AID^{50}. See Fig. 6.
		V2						0/5			0/5	1/5	3/5	
		V3							0/5		0/5	0/5	3/5	
		V4								0/5	0/5	2/5	3/5	
		S1					5/5				5/5	0/5	0/5	
		S2						3/5			2/5	0/5	0/5	
		S3							4/5		4/5	0/5	0/5	
		S4								1/5	1/5	0/5	0/5	

*The 6th passage was used at 2, 5, 7 and 11 days, and the 8th passage at 21 days, after vaccination.

by a serological response. Since autopsy of its particular group was performed only six days after challenge, it is probable that the lack of response was due simply to lack of time.

DISCUSSION

The observation that immunity follows infection with *M. gallisepticum* is not new. The value of the present work lies more in the demonstration of the relatively small number of organisms required to provoke it, the rapidity of its onset and its well-nigh impregnable character. In addition, it gives fresh insight into the true nature of the disease caused by *M. gallisepticum.*

In Experiment 1, the choice of dilutions 10^{-5} and 10^{-6} for challenge was fortunate in that they provided an adequate, but not too severe, test of immunity. The higher dilution, being so close to 1 AID_{50}, was a useful one for making at least an approximate estimate of the MID. In Experiment 2, the dilutions selected could have been more satisfactory, especially in view of the fact that one of the objectives, measurement of the degree of immunity, was not really achieved. However, this does serve to illustrate to what extent it is necessary to resort to guesswork in such experiments and underlines the need for a rapid method to estimate the concentration of *Mycoplasma* in culture, a procedure which at the moment takes several days. As a further example, it was seven days after the experiment was commenced before it could be known that the size of the vaccinating dose was approximately 10^8 organisms. This dose was at least 10 times greater than had been intended, since at a similar level in Experiment 1, coryza had developed and 1 out of 8 birds was found to have air-sac lesions at autopsy. This information, combined with the incomplete immunity found after the 1st, 2nd and 3rd challenges, was quite disturbing as the immunising dose might well have been excessive. In each of the first 4 treatments challenge, even at the lowest level, was too severe, although it was still possible to observe the presence of immunity at five days post-vaccination. However, the lack of complete protection at seven days was an indication to pause after challenge at eleven days and consider the results before proceeding with the 5th challenge. It was found that not all individuals could withstand approximately 100 AID_{50}, so for the final challenge, dilutions were selected to give approximately 1,000, 100, 10 and 1 AID_{50}, per ml. Unfortunately, the greatest challenge turned out to be in the region of 300 AID_{50}, and since complete protection was obtained at this level, the maximum degree of immunity was not found.

There have been many demonstrations that, when faced with the defence mechanisms of the healthy chicken, *M. gallisepticum* has little inherent ability to extend much beyond the nasal cavity (Grumbles, Boney, and Delaplane, 1952; Nelson, 1953; Fahey and Crawley, 1956; Chu and Newnham, 1959). Nevertheless, in the last decade, this organism has come to be regarded as a major factor in

the production of exudative tracheitis and caseous air-sac lesions. The evidence for this belief lies in the frequency with which *M. gallisepticum* can be isolated from these disease processes, which in turn can be reproduced by its intratracheal or intraperitoneal inoculation, into susceptible chickens. It might be theorized then, that in natural infection of the lower respiratory tract the organism has received assistance from other sources (an opinion held by many workers), or by-passed the upper respiratory defences by means of egg transmission.

A reason for the inability of *M. gallisepticum* to progress from the nasal cavity to the air-sacs is provided by the present work. Here it has been shown that infection of the nasal mucosa results in the production of an immunity, apparent in five days or less, and by 21 days enabling chickens to withstand more than 300 AID_{50}. What this latter challenge represents in terms of natural exposure is difficult to estimate. Some idea may be gained from the fact that 10^8 organisms (approximately one million AID_{50}) instilled into the nasal passages of 8 susceptible chickens, produced air-sac lesions in only one; cultures and histological sections from the other 7 birds indicated that infection had not even reached the trachea.

The single instance of lower respiratory disease mentioned above is worthy of further consideration. In the absence of any known enhancing factor, it is postulated that organisms were inhaled deep into the respiratory tract during intranasal instillation, or shortly thereafter, producing lower respiratory disease before the development of immunity. The theory is substantiated, as already described in Experiment 1 by the histological finding that the process had been in progress for some time. The interval between intranasal infection and autopsy was actually 25 days.

This, of course, may not be the whole explanation as it is notoriously difficult with *Mycoplasma* infections to reproduce the natural disease experimentally. Indeed the mode of transmission is in most species unknown and, as in the case of *M. gallisepticum*, the influence of an "assisting" factor or factors is postulated (Klieneberger-Nobel, 1954). In bovine pleuropneumonia, cattle exposed by the respiratory route rarely develop lung lesions unless the infecting culture is in an extremely fine state of division. In view of the results reported here, it would be interesting indeed to test the immunity of cattle simply inoculated intranasally. Some evidence that these animals could be immune has been provided by Cordy and Adler (1960) who used a *Mycoplasma* isolated from goats to infect sheep and found that survivors of challenge by aerosol and skin scarification subsequently resisted intradermal inoculation, which route of exposure had previously proved highly effective in producing lesions. However, the picture is largely incomplete as no challenge controls were available, and the serological results are not informative.

Since the true origin of the resistance of the air-sacs against

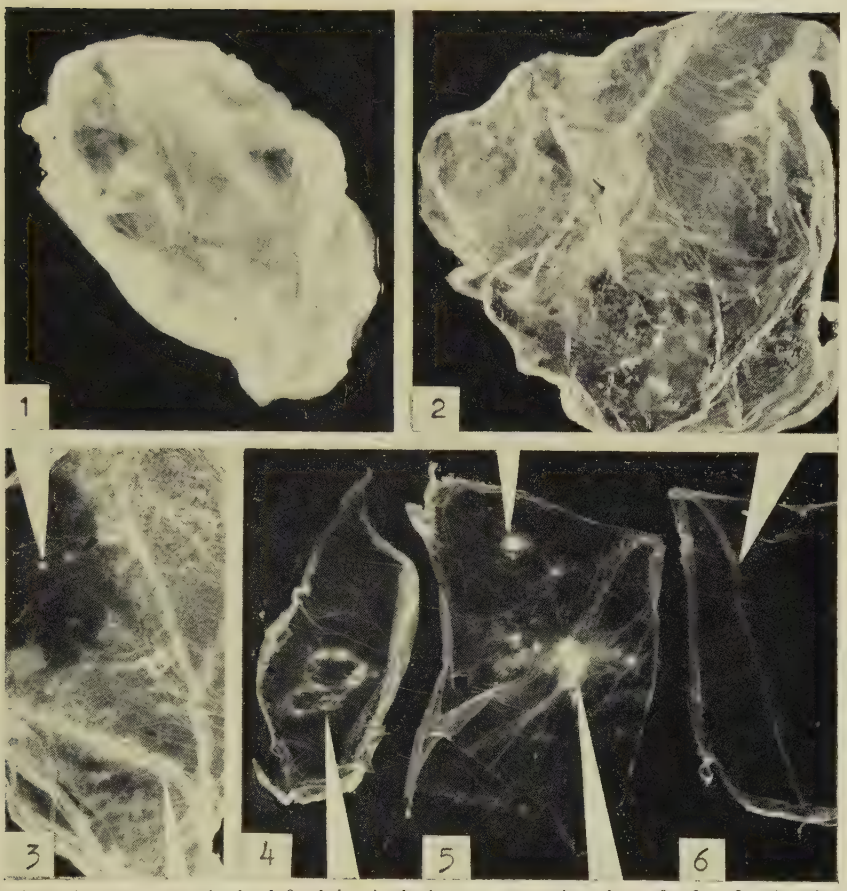
infection with *M. gallisepticum* is as yet unknown, the nasal cavity at the moment may receive the credit. Histological examination reveals the close correlation between reactivity in this area and subsequent immunity. The cellular reaction is largely lymphocytic in nature and, there being already present in the lamina propria of the "normal" chicken a considerable accumulation of these cells, one wonders to what extent this is related to the rapid appearance of immunity. Their strategic situation might also account for the swift serological response. In Experiment 2, blood samples taken at 2 days after intranasal exposure were negative for agglutinins. Subsequently, of 5 samples taken one was positive, on the 3rd day, two on the 4th and by the 7th all were reacting with titres up to 1:40. It is not intended to suggest, however, that agglutinating antibodies are responsible for immunity. One attempt to demonstrate passive immunity, using antiserum produced in chickens with an agglutination titre of 1:640, gave no indication that immune serum per se was of any protective value.

The failure of immune serum to prevent disease leads to speculation on the nature of the resistance. Histological examination of the air-sacs from immune and normal chickens does not reveal any difference. The organism certainly remains in the nasal cavity for a lengthy period and, although the duration of immunity is still unknown, an obvious possibility is the well-known situation in which immunity lasts only as long as persistence of infection. If in fact this is the case, difficulties can be forseen in the use of this technique for intranasal vaccination on a large scale. Immunity may be broken down by concurrent infections or an unfriendly environment, since these often appear to be associated with the conversion of the relatively mild coryza of *M. gallisepticum* to an intractable, generalised respiratory affliction. The use of a small immunising dose, or of a strain of *Mycoplasma* less virulent than the S6, might reduce the possibility of complications. Breed susceptibility, often discussed but never specifically proved, may be an important consideration. However, if active air-sac lesions can be prevented, the transmission of *M. gallisepticum* from breeding stock to their progeny via the hatching egg seems remote. Its use in growing birds in conjunction with the present-day broadside of other vaccines, could not be condoned. The turkey, although more susceptible than the chicken to *Mycoplasma* infection, may be a more suitable subject for vaccination since it is apparently much less liable to complicating respiratory diseases.

CONCLUSIONS

Intranasal instillation of 10^3 or more virulent *M. gallisepticum* produced immunity in chickens against homologous challenge in the abdominal air-sac.

After exposure to approximately 10^8 organisms, immunity was



Tissue in every case is the left abdominal air-sac, or portion thereof, after fixation for several days in 10 per cent formalin. × 1·5.

Fig. 1. Susceptible chicken. Air-sac 14 days after exposure to approximately 10^2 viable *M. gallisepticum.*

Fig. 2. Challenge with approximately 10^5 organisms 5 days after immunisation. Diminution in extent of lesions.

Fig. 3. Challenge with approximately 10^6 organisms 7 days after immunisation. Marked reduction in severity of lesions, with some areas of complete localisation; most striking reaction appears around blood vessels.

Fig. 4. Challenge with approximately 10^6 organisms 11 days after immunisation produced only local lesions.

Fig. 5. Scars at sites of local invasion in air-sac from immune chicken, 7 days after challenge with approximately 10^4 organisms.

Fig. 6. Challenge with approximately 10^5 organisms 21 days after immunisation. Apart from slight opacity around blood vessel, the tissue is normal.

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demonstrated by the 5th day; on the 21st day, chickens resisted more than 300 AID_{50}.

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